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**(54) Phytase formulation**

(57) A stabilized enzyme formulation is disclosed which comprises phytase and at least one stabilizing agent selected from the group consisting of:

- a) C<sub>5</sub> sugars such as xylitol and ribitol,
- b) polyethylene glycol having a molecular weight of 600 to 4000 Da,
- c) the disodium salts of malonic, succinic and glutaric acid, and
- d) carboxymethylcellulose, and
- e) sodium alginate.

Alternatively, phytase may be stabilized by chemical crosslinking with either

- a) glutaraldehyde, or
- b) oxidation of phytase carbohydrate residues with sodium periodate and subsequent addition of adipic acid dihydrazide.

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## Description

[0001] The present invention relates to liquid and dry phytase formulations having an increased stability, preferably thermostability, which is obtained by the addition of stabilizing agents, or by crosslinking.

[0002] Although a large amount of phosphate is present in feed in form of phytate phosphorus, monogastric animals, like pigs and poultry, lack the ability to use this form of phosphate. The alkali or earth alkali salts of phytic acid occur naturally mainly in cereals. Since monogastric animals are not able to use this form of phosphate it is common practice to add phosphate to animal feed.

[0003] On the other hand an enzyme called phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is known to occur in plants and in some microorganisms. Since phytase can be produced by fermentation it is known in the art to use phytase as an animal feed additive in order to enhance the nutritive value of plant material by liberation of inorganic phosphate from phytic acid (*myo*-inositol hexakisphosphate). By adding phytase to the animal feed the level of phosphorus pollution of the environment can be reduced since the animal is able to make use of the phosphate liberated from phytate by the use of phytase.

[0004] For feed application a stable preferably thermostable phytase is of general interest in order to avoid problems that may occur during the formulation (e.g. spray drying, granulation) and feed treatment processes (e.g. pelleting, extrusion, expansion) where temporarily high temperatures (up to 80-120 °C) and shear stress may affect the protein structure and lead to an undesired loss of activity.

[0005] The international patent application WO 93/16175 of Gist-Brocades describes stabilized liquid formulations of phytase. It is suggested to use as stabilizing agent urea and a water-soluble polyol whereby sorbitol, glycerol and polyethylene glycol having a molecular weight of 6000 are mentioned.

[0006] It is an object of the present invention to improve the stability, preferably thermostability of phytase whereby stability is defined as the ability to retain activity under various conditions. This stability aspect relates to the entire life cycle of the enzyme which comprises production (fermentation, downstream processing, formulation and heat treatment of feed), distribution (transport and storage) and final application. For a commercially interesting enzyme like phytase it is important to withstand the high temperatures reached during various feed treatment processes like pelleting, extrusion and expansion (up to 80-120 °C) and to be stable during long-term storage.

[0007] The term "stability" as used in the present invention relates to all the specifications of an industrial enzyme which comprise aspects such as activity, specificity, shelf stability, mechanical stability, microbial stability, toxicity, chemical composition and physical parameters such as density, viscosity, hygroscopy, but also colour, odour and dust. A preferred aspect of the present invention relates to the stability of phytase against thermal inactivation during formulation and feed treatment processes such as pelleting, extrusion and expansion.

[0008] A major barrier to the wide use of phytases is the constraint of thermal stability (80-120 °C) required for these enzymes to withstand inactivation during feed treatment processes. The currently available industrial phytases all originate from *A. niger* and have a low intrinsic resistance to heat inactivation. As an alternative or in addition to molecular biological approaches the present invention enhances the stability, preferably thermostability of a protein by the addition of different additives and in another aspect by the chemical crosslinking of enzyme monomers to oligomers.

[0009] The experiments leading to the present invention were also performed with the so-called consensus phytase, a phytase developed according to a theoretical molecular biological approach which has a higher intrinsic stability compared with *Aspergillus* phytases, see European Patent Application Publication No. 897 985. In the practice of the present invention the consensus phytases specifically described in examples 3 - 13 can also be used.

[0010] The present invention discloses the use of different additives which act as stabilizing agent on the stability, preferably thermostability of the enzyme.

[0011] Regarding the temperature dependence of the specific activity of the non-formulated phytases which can preferably be used in the present invention three different groups can be formed according to their activity maximum. The activity maximum is reached at the following temperatures: for *A. fumigatus* and *A. niger* phytase at 55 °C, for *A. terreus* CBS and *A. nidulans* phytase at 45 °C and for consensus phytase at 65 °C. A temperature of 10-15 °C above the determined temperature maximum - where the non-formulated phytases were completely inactive - was chosen as screening point for studying the effect of the stabilizing agents on the thermostability of phytases, i.e. 60 °C for *A. nidulans* and *A. terreus* CBS phytase, 65 °C for *A. niger* and *A. fumigatus* phytase, and 75 °C for consensus phytase.

[0012] The present invention provides a stabilized, preferably thermostabilized enzyme formulation comprising phytase and at least one stabilizing agent selected from the group consisting of:

a) polyols containing five carbon atoms, preferably C<sub>5</sub> sugars, more preferably xylitol or ribitol,

b) polyethylene glycol having a molecular weight of 600 to 4000 Da,

c) the disodium salts of malonic, glutaric and succinic acid,

d) carboxymethylcellulose, and

e) sodium alginate

5 [0013] The present invention also provides a stabilized, preferably thermostabilized enzyme formulation comprising phytases which have been crosslinked:

a) by chemical reactions with glutaraldehyde; or by

b) oxidation with sodium periodate and subsequent addition of adipic acid dihydrazide

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[0014] Although it would be possible to use other phytases obtained from other sources than microorganisms it is preferred to use a phytase which has been produced by microorganisms. In the present invention preferably such phytases are used which are produced by a fungus, and more preferably from the group consisting of *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus terreus*, and *Aspergillus niger*. Another phytase preferably used in this invention is the so called consensus phytase. It is, however, also possible to produce such phytases by genetic engineering whereby the gene obtained from a fungus is transferred to a host organism like a bacterium (e.g. *E. coli*), a yeast or another fungus, for further details, see e.g. European Patent Application Publication No. 684313 and European Patent Application Publication No. 897 010.

[0015] The term enzyme formulation comprises all liquid and dry formulations in which the enzyme phytase may be commercialized. Preferably, the source of phytase for such a formulation is a rather raw, liquid preparation obtained from the fermentation broth. For the preparation of a liquid phytase formulation the selected stabilizing agents are added or the phytase is crosslinked. To obtain a stabilized, preferably thermostabilized dry formulation the phytase is a) spray dried or granulated in the presence of the selected stabilizing agents, or b) chemically crosslinking.

[0016] In one preferred embodiment the liquid enzyme formulation comprises as stabilizing agent polyethylene glycol whereby the polyethylene glycol is present in a concentration of 10-50% (w/w) in the final formulation.

[0017] Preferably the enzyme formulation comprises polyethylene glycol having a molecular weight of 1000-3350 Da. It is especially preferred to use a polyethylene glycol having a molecular weight of about 1450. Polyethylene glycols with molecular weights slightly outside of the preferred range (600 Da and 4000 Da, respectively) showed still reasonable effect but are less preferred. The stabilizing effect of polyethylene glycol was shown to be molecular weight-dependent (see Figures 2 and 3).

[0018] In another preferred embodiment of the present invention the stabilizing agent is xylitol or ribitol. Both are sugar alcohols having a five carbon atom structure. Xylitol and ribitol are preferably used in a concentration of 20 to 60% (w/w) in the final liquid formulation. Surprisingly xylitol and ribitol as stabilizing agents of, e.g., *A. fumigatus* phytase increased the specific activity measured at 65 °C to 11-12 U/mg at a concentration of 12.5%, and to 51-90 U/mg at a concentration of 25% of the polyol (see Figure 4).

[0019] In another embodiment of the present invention the liquid enzyme formulation comprises as stabilizing agent the disodium salts of glutaric, succinic or malonic acid whereby the concentration of the salt in the final formulation ranges between 10 and 30% (w/w). The addition of malonate, succinate and glutarate at a concentration of 25% resulted in a significant increase in *A. fumigatus* phytase thermostability with considerable activity still being detected at 70 °C for malonate and 65 °C for succinate and glutarate as can be seen in Figure 6.

[0020] In addition thereto the carboxylates stimulated *A. fumigatus* phytase activity measured at 37 °C with an approximately 4-fold increase in phytase activity being observed in the case of malonate, a 2-fold increase for succinate and minor effects for glutarate. Investigation of different concentrations (5, 10 and 25%) of malonate showed that thermostabilization of *A. fumigatus* phytase is concentration-dependent whereas stimulation of enzymatic activity, at least in this concentration range, is not (see Figure 7). In contrast to these findings different concentrations (5, 10 and 25%) of sodium acetate which is a monocarboxylic acid, caused an up to 2-fold increase in specific activity of *A. fumigatus* phytase at 37 °C, but had only minor effects on the thermostability of the protein (see Figure 8). Therefore, it may be concluded that carboxylate groups are responsible for activity modulation whereas bifunctional dicarboxylates stabilize phytases possibly by ionic interactions. The sodium malonate and succinate generally increased the thermostability of *A. nidulans*, *A. terreus* CBS, *A. niger* and consensus phytase by 5-15 °C. On the other hand stimulation of phytase activity was only observed for *A. nidulans* and *A. fumigatus* phytase both having rather low specific activity but not for *A. terreus* CBS, *A. niger* and consensus phytase (see Figures 9 and 10).

[0021] In another embodiment of the present invention the enzyme formulation comprises as stabilizing agent the polymers carboxymethylcellulose and/or sodium alginate whereby the concentration in the final liquid formulation is between 1 and 20% preferably 1 and 10% (w/w). The addition of these polymeres to *A. fumigatus* phytase preparations resulted in a significant 5 to 10% increase of phytase thermostability.

[0022] In another embodiment of the present invention the enzyme formulation comprises as stabilizing agent alginate, preferably sodium alginate and most preferably in a concentration of 1 to 10% (w/w) in the final liquid formulation.

[0023] In a further embodiment of the present invention the enzyme formulation comprises crosslinked phytase. For the preparation of such a stabilized phytase form, glutaraldehyde is added to the phytase at a concentration resulting in an oligomerization of the protein.

[0024] In another embodiment the enzyme formulation comprises phytase which has been crosslinked via its carbohydrate chains. Crosslinking involves as a first step periodate oxidation of the carbohydrate residues followed by reaction of the generated aldehyde groups with adipic acid dihydrazide.

[0025] Depending on the conditions employed, the crosslinking reaction can lead to various derivatives of an enzyme, namely

a) modified enzyme molecules that have reacted with only one hydrazide group of adipic acid dihydrazide,

b) intramolecularly crosslinked enzymes, with or without intermolecular crosslinking, and

c) intermolecularly crosslinked, soluble oligomers or insoluble polymers.

[0026] In most cases the reaction results in a mixture of several forms. Crosslinking of *A. fumigatus* and consensus phytase both expressed in *Hansenula polymorpha* resulted in the formation of oligomeric forms. The degree of crosslinking could be controlled effectively by changing the degree of enzyme oxidation. An optimal thermostabilization of phytase has been observed at a concentration of 50 mM sodium periodate applied to a 5 mg/ml phytase solution. For both phytases an increase in thermostability between 10 and 15 °C has been observed (see Figure 12). It should be noted that the oxidized phytases formed a significant amount of dimers, trimers and tetramers even without addition of adipic acid dihydrazide (see Figure 11A).

[0027] Another aspect of the present invention concerns the use of the listed stabilizers as additives for the production of dry/solid phytase formulations. In this embodiment of the present invention the addition of stabilizers (1 to 20% (w/w) of xylitol/ribitol, 1 to 20% (w/w) of polyethylene glycols with a molecular weight preferably between 1000 and 3350 Da and/or 1 to 20% (w/w) of dicarboxylates like malonate, succinate and glutarate, and/or 1 to 10% (w/w) of the polymers carboxymethylcellulose and/or alginate, preferably sodium alginate dissolved in 100-200 ml phytase liquid (crosslinked or non-crosslinked) or added as solid compounds to the standard granulation mixture (containing ligninsulfonate as binder, silica and gypsum as carrier) Such formulation can result in an increased recovery (up to 20%) of phytase activity determined after a high shear granulation process which included a drying step of the granulates on a fluid bed dryer at 45°C for 15 mm. In addition such granulates which contain stabilizers can show, when mixed with feed, an increased recovery of enzymatic activity after the feed treatment (e.g. a pelleting process at 85°C) compared to granulates without such additives.

[0028] Another aspect of the present invention concerns methods of preparing feed compositions for monogastric animals, whereby the feed is supplemented with a thermostabilized dry or liquid enzyme formulation according to any of claims (1-13). The phytase supplemented feed can be subjected on several methods of feed processing like extrusion, expansion and pelleting, where temporarily high temperatures may occur and thermostabilization is an advantage.

[0029] The stabilized enzyme formulation of the present invention can be applied for example on feed pellets. The thermostabilized liquid enzyme formulation may be diluted with tap water to yield a solution having the desired activity of phytase (100 - 200 phytase units/g solution). The feed pellets can be transferred to a mechanical mixer and the diluted enzyme formulation is sprayed onto the feed pellets while being agitated in order to yield a homogeneous product with an added phytase activity of for example 500 units phytase/kg feed pellets. Alternatively the dry or liquid enzyme formulation can be directly mixed with the mash feed before this mixture is then subjected to a process such as pelleting, expansion or extrusion.

[0030] In a further aspect the present invention concerns a method of providing a monogastric animal with its dietary requirement of phosphorus wherein the animal is fed with a feed according to the present invention and whereby no additional phosphorus is added to the feed.

[0031] The results of the experiments of the present invention are shown in the following Figures.

**Figure 1.** Comparison of the temperature dependence of activity of *A. fumigatus*, *A. nidulans*, *A. terreus* CBS, *A. niger* and consensus phytase measured under standard assay conditions as described in Example 1.

**Figure 2.** Effect of different polyethylene glycols on the specific activity of *A. fumigatus* phytase at 65 °C.

**Figure 3.** Effect of 50% solutions of polyethylene glycols with different molecular weights on the thermostability of *A. niger*, consensus, *A. terreus* CBS and *A. nidulans* phytase. The specific activities were measured at 60 °C for *A. terreus* CBS and *A. nidulans* phytase, at 65 °C for *A. niger* phytase and at 75 °C for consensus phytase.

Figure 4. Effect of 25 and 50% solutions of different polyols on the specific activity of *A. fumigatus* phytase at 65 °C.

Figure 5. Temperature dependence of activity of *A. niger* (A), consensus (B), *A. nidulans* (C) and *A. terreus* CBS (D) phytase in the presence of 50% xylitol as additive.

Figure 6. Temperature dependence of activity of *A. fumigatus* phytase in the presence of 25% concentrations of disodium malonate, succinate and glutarate.

Figure 7. Temperature dependence of activity of *A. fumigatus* phytase in the presence of 5, 10 and 25% disodium malonate.

Figure 8. Temperature dependence of activity of *A. fumigatus* phytase in the presence of 5, 10 and 25% sodium acetate.

Figure 9. Temperature dependence of activity of *A. niger* (A), consensus (B), *A. terreus* CBS (C) and *A. nidulans* (D) phytase in the presence of 25% disodium malonate.

Figure 10. Temperature dependence of activity of *A. niger* (A), consensus (B), *A. terreus* CBS (C) and *A. nidulans* (D) phytase in the presence of 25% disodium succinate.

Figure 11.

A) SDS-PAGE of *A. fumigatus* phytase samples after incubation with different concentrations of sodium periodate.

B) SDS-PAGE of the different oxidized *A. fumigatus* phytase samples from (A) after subsequent crosslinking with adipic acid dihydrazide.

Figure 12 Temperature dependence of activity of *A. fumigatus* phytase and consensus phytase before and after crosslinking with periodate/adipic acid dihydrazide.

Figure 13 Design of the consensus phytase sequence. The letters represent the amino acid residues in the one-letter code. The following sequences were used for the alignment: *phyA* from *Aspergillus terreus* 9A-1 (Mitchell et al, 1997; from amino acid (aa) 27), *phyA* from *A. terreus* cbs116.46; (van Loon et al., 1998; from aa 27), *phyA* from *Aspergillus niger* var. *awamori* (Piddington et al, 1993; from aa 27), *phyA* from *A. niger* T213; from aa 27), *phyA* from *A. niger* stain NRRL3135 (van Hartingsveldt et al, 1993; from aa 27), *phyA* from *Aspergillus fumigatus* ATCC 13073 (Pasamontes et al, 1993; from aa 25), *phyA* from *A. fumigatus* ATCC 32722 (van Loon et al, 1998; from aa 27), *phyA* from *A. fumigatus* ATCC 58128 (van Loon et al., 1998; from aa 27), *phyA* from *A. fumigatus* ATCC 26906 (van Loon et al, 1998; from aa 27), *phyA* from *A. fumigatus* ATCC 32239 (van Loon et al, 1998; from aa 30), *phyA* from *Emmericella nidulans* (Pasamontes et al, 1997a; from aa 25), *phyA* from *Talaromyces thermophilus* (Pasamontes et al, 1997a; from aa 24), and *phyA* from *Myceliophthora thermophila* (Mitchell et al, 1997; from aa 19). The alignment was calculated using the program PILEUP. The location of the gaps was refined by hand. Capitalized amino acid residues in the alignment at a given position belong to the amino acid coalition that establish the consensus residue. In bold, beneath the calculated consensus sequence, the amino acid sequence of the finally constructed consensus phytase (Fcp) is shown. The gaps in the calculated consensus sequence were filled by hand according to principals stated in Example 3.

Figure 14 DNA sequence of the consensus phytase-1 gene (*fcp*) and of the primers used for the gene construction. The calculated amino acid sequence (Figure 13) was converted into a DNA sequence using the program BACKTRANSLATE (Devereux et al., 1984) and the codon frequency table of highly expressed yeast genes (GCG program package, 9.0). The signal peptide of the phytase from *A. terreus* cbs.116.46 was fused to the *N*-terminus. The bold bases represent the sequences of the oligonucleotides used to generate the gene. The names of the respective oligonucleotides are alternately noted above or below the sequence. The underlined bases represent the start and stop codon of the gene. The bases written in italics show the two introduced *Eco* RI sites.

Figure 15 Alignment and consensus sequence of five *Basidiomycetes* phytases. The letters represent the amino acid residues in the one-letter code. The amino acid sequences of the phytases from *Paxillus involutus*, *phyA1* (aa 21) and *phyA2* (aa 21, WO 98/28409), *Trametes pubescens* (aa 24, WO 98/28409), *Agrocybe pediades* (aa 19,

WO 98/28409), and *Peniophora lycii* (aa 21, WO 98/28409) starting with the amino acid residues mentioned in parentheses, were used for the alignment and the calculation of the corresponding consensus sequence called "Basidio" (Example 4). The alignment was performed by the program PILEUP. The location of the gaps was refined by hand. The consensus sequence was calculated by the program PRETTY. While a vote weight of 0.5 was assigned to the two *P. involutus* phytases, all other genes were used with a vote weight of 1.0 for the consensus sequence calculation. At positions, where the program was not able to determine a consensus residues, the Basidio sequence contains a dash. Capitalized amino acid residues in the alignment at a given position belong to the amino acid coalition that establish the consensus residue.

**Figure 16** Design of consensus phytase-10 amino acid sequence. Adding the phytase sequence of *Thermomyces lanuginosa* (Berka *et al.*, 1998) and the consensus sequence of the phytases from five *Basidiomycetes* to the alignment of Figure 13, an improved consensus sequence was calculated by the program PRETTY. Additionally, the amino acid sequence of *A. niger* T213 was omitted, therefore, using a vote weight of 0.5 for the remaining *A. niger* phytase sequences. For further information see Example 14.

**Figure 17** DNA and amino acid sequence of consensus phytase-10. The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The sequence of the oligonucleotides which were used to assemble the gene are in bold letters. The label of oligonucleotides and the amino acids, which were changed compared to those for consensus phytase -1, are underlined and their corresponding triplets are highlighted in small cases. The *fcp10* gene was assembled from the following oligonucleotides: CP-1, CP-2, CP-3.10, CP-4.10, CP-5.10, CP-6, CP-7.10, CP-8.10, CP-9.10, CP-10.10, CP-11.10, CP-12.10, CP-13.10, CP-14.10, CP-15.10, CP-16.10, CP-17.10, CP-18.10, CP-19.10, CP-20.10, CP-21.10, CP-22.10. The newly synthesized oligonucleotides are additionally marked by number 10. The phytase contains the following 32 exchanges: Y54F, **E58A**, D69K, D70G, A94K, N134Q, I158V, S187A, Q188N, **D197N**, S204A, T214L, D220E, L234V, A238P, D246H, T251N, Y259N, **E267D**, E277Q, A283D, **R291I**, A320V, **R329H**, **S364T**, I366V, **A379K**, S396A, **G404A**, Q415E, A437G, A463E. The mutations accentuated in bold letters revealed a stabilizing effect on consensus phytase-1 as tested as single mutation in consensus phytase-1.

**Figure 18** Alignment for the design of consensus phytase-11. In contrast to the design of consensus phytase-10, for the design of the amino acid sequence of consensus phytase-11, all *Basidiomycetes* phytases were used as independent sequences using an assigned vote weight of 0.2 for each *Basidiomycetes* sequence. Additionally, the amino acid sequence of *A. niger* T213 was used in that alignment, again.

**Figure 19** DNA and amino acid sequence of consensus phytase-1-thermo[8]-Q50T-K91A. The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The replaced amino acid residues are underlined. The stop codon of the gene is marked by a star (\*).

**Figure 20** DNA and amino acid sequence of consensus phytase-10-thermo[3]-Q50T-K91A. The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The replaced amino acid residues are underlined. The stop codon of the gene is marked by a star (\*).

**Figure 21** DNA and amino acid sequence of *A. fumigatus* ATCC 13073 phytase a-mutant. The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The replaced amino acid residues are underlined. The stop codon of the gene is marked by a star (\*).

**Figure 22** DNA and amino acid sequence of consensus phytase-7. The amino acids are written above the corresponding DNA sequence using the one-letter code. The sequence of the oligonucleotides used to assemble the gene are in bold letters. Oligonucleotides and amino acids that were exchanged are underlined and their corresponding triplets are highlighted in small cases. The *fcp7* gene was assembled from the following oligonucleotides: CP-1, CP-2, CP-3, CP-4.7, CP-5.7, CP-6, CP-7, CP-8.7, CP-9, CP-10.7, CP-11.7, CP-12.7, CP-13.7, CP-14.7, CP-15.7, CP-16, CP-17.7, CP-18.7, CP-19.7, CP-20, CP-21, CP-22. The newly synthesized oligonucleotides are additionally marked by number 7. The phytase contains the following 24 exchanges in comparison to the original consensus phytase: S89D, S92G, A94K, D164S, P201S, G203A, G205S, H212P, G224A, D226T, E255T, D256E, V258T, P265S, Q292H, G300K, Y305H, A314T, S364G, M365I, A397S, S398A, G404A, and A405S.

**Figure 23** Differential scanning calorimetry (DSC) of consensus phytase-1 and consensus phytase-10. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10 °C/min was applied up to 95 °C. DSC of consensus phytase-10 (upper graph) yielded

a melting temperature of 85.4 °C, which is 7.3 °C higher than the melting point of consensus phytase-1 (78.1 °C, lower graph).

**Figure 24** Differential scanning calorimetry (DSC) of consensus phytase-10-thermo-Q50T and consensus phytase-10-thermo-Q50T-K91A. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10 °C/min was applied up to 95 °C. DSC of consensus phytase-10-thermo-Q50T (upper graph) yielded a melting temperature of 88.6 °C, while the melting point of consensus phytase-10-thermo-Q50T-K91A was found at 89.3 °C.

**Figure 25** Comparison of the temperature optimum between consensus phytase-1, consensus phytase-10 and consensus phytase-10-thermo-Q50T. For the determination of the temperature optimum, the phytase standard assay was performed at a series of temperatures between 37 and 86 °C. The diluted supernatant of transformed *S. cerevisiae* strains was used for the determination. The other components of the supernatant showed no influence on the determination of the temperature optimum:  $\wedge$ , consensus phytase-1;  $\diamond$ , consensus phytase-10;  $\blacksquare$ , consensus phytase 10-thermo-Q50T.

**Figure 26** pH-dependent activity profile and substrate specificity of consensus phytase-10 and its variants thermo-Q50T and thermo-Q50T-K91A. The phytase activity was determined using the standard assay in appropriate buffers (see Example 11) at different pH-values. Graph a) shows the pH-dependent activity profile of consensus phytase-10 ( $\square$ ), consensus phytase-10-thermo-Q50T ( $\cdot$ ), and consensus phytase-10-thermo-Q50T-K91A ( $\wedge$ ). Graph b) shows the corresponding substrate specificity tested by replacement of phytate by the indicated compounds in the standard assay; open bars, consensus phytase-10 (grey bars, consensus phytase-10-thermo-Q50T; dark bars, consensus phytase-10-thermo-Q50T-K91A). The numbers correspond to the following compounds: 1, phytate; 2, *p*-nitrophenyl phosphate; 3, phenyl phosphate; 4, fructose-1,6-bisphosphate; 5, fructose-6-phosphate; 6, glucose-6-phosphate; 7, ribose-5-phosphate; 8, DL-glycerol-3-phosphate; 9, glycerol-2-phosphate; 10, 3-phosphoglycerate; 11, phosphoenolpyruvate; 12, AMP; 13, ADP; 14, ATP.

**Figure 27** pH-dependent activity profile and substrate specificity of consensus phytase-1-thermo[8]-Q50T and of consensus phytase-1-thermo[8]-Q50T-K91A. The phytase activity was determined using the standard assay in appropriate buffers (see Example 11) at different pH-values. Graph a) shows the pH-dependent activity profile of the Q50T- ( $\blacksquare$ ) and the Q50T-K91A-variant ( $\cdot$ ). Graph b) shows the corresponding substrate specificities tested by replacement of phytate by the indicated compounds in the standard assay (open bars, consensus phytase-1-thermo[8]-Q50T; filled bars, consensus phytase-1-thermo[8]-Q50T-K91A). The substrates are listed in the legend of Figure 26.

**Figure 28** Differential scanning calorimetry (DSC) of consensus phytase-1-thermo[8]-Q50T and consensus phytase-1-thermo[8]-Q50T-K91A. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10 °C/min was applied up to 95 °C. DSC of consensus phytase-1-thermo[8]-Q50T (upper graph) showed a melting temperature of 84.7 °C, while the melting point of consensus phytase-1-thermo[8]-Q50T-K91A was found at 85.7 °C.

**Figure 29** Comparison of the temperature optimum between consensus phytase-1, consensus phytase-1-thermo[3] and consensus phytase-1-thermo[8]. For the determination of the temperature optimum, the phytase standard assay was performed at a series of temperatures between 37 and 86 °C. Purified protein from the supernatant of transformed *S. cerevisiae* strains was used for the determination.  $\bigcirc$ , consensus phytase-1;  $\square$ , consensus phytase-1-thermo[3];  $\blacktriangle$ , consensus phytase 1-thermo[8].

**Figure 30** Comparison of the pH-dependent activity profile and substrate specificity of consensus phytase-1, consensus phytase-7, and of the phytase from *A. niger* NRRL 3135. The phytase activity was determined using the standard assay in appropriate buffers (see Example 11) at different pH-values. Graph a) shows the pH-dependent activity profile of consensus phytase-1 ( $\blacksquare$ ), the phytase from *A. niger* NRRL 3135 ( $\bigcirc$ ), and of consensus phytase-7 ( $\blacktriangle$ ). Graph b) shows the corresponding substrate specificity tested by replacement of phytate by the indicated compounds in the standard assay (black bars, *A. niger* NRRL 3135 phytase; grey bars, consensus phytase-1, dashed bars, consensus phytase-7). The substrates are listed in the legend of Figure 26.

**Figure 31** Differential scanning calorimetry (DSC) of the phytase from *A. fumigatus* ATCC 13073 and of its stabilized  $\alpha$ -mutant, which contains the following amino acid exchanges F55Y, V100I, F114Y, A243L, S265P, N294D. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium ace-

tate, pH 5.0. A constant heating rate of 10 °C/min was applied up to 95 °C. DSC of consensus *A. fumigatus* 13073 phytase (upper graph) revealed a melting temperature of 62.5 °C, while the melting point of the  $\alpha$ -mutant was found at 67.0 °C

**Figure 32** Comparison of the temperature optimum of *A. fumigatus* 13073 wild-type, its *A. fumigatus*  $\alpha$ -mutant, and a further stabilized  $\alpha$ -mutant (E59A-S126N-R329H-S364T-G404A). For the determination of the temperature optimum, the phytase standard assay was performed at a series of temperatures between 37 and 75 °C. The diluted supernatant of transformed *S. cerevisiae* strains was used for the determination. The other components of the supernatant showed no influence on the determination of the temperature optimum. ○, *A. fumigatus* ATCC 13073 phytase; ▲, *A. fumigatus* ATCC 13073  $\alpha$ -mutant; □, *A. fumigatus* ATCC 13073  $\alpha$ -mutant-(E59A-S126N-R329H-S364T-G404A)-Q27T; ■, *A. fumigatus* ATCC 13073  $\alpha$ -mutant-(E59A-S126N-R329H-S364T-G404A)-Q27T-K68A. Q27T and K68A corresponds to consensus phytase-1 Q50T and K91A, respectively.

**Figure 33** Amino acid sequence of consensus phytase 12 (consphy12) which contains a number of active site residues transferred from the "basidio" consensus sequence to consensus phytase-10-thermo-Q50T-K91A.

### Example 1

#### a) Materials

[0032] Phytic acid (dodecasodium salt) and polyethylene glycols, polyols, sodium dicarboxylates, sodium periodate, adipic acid dihydrazide and other additives were purchased from commercial suppliers. All other chemicals were at least of analytical grade. Five-ml HiTrap desalting columns were obtained from Pharmacia. SDS-PAGE gels (4-12% NuPAGE Bis-Tris Pre-Cast) and buffers were delivered by NOVEX.

#### b) Expression and purification of phytases

[0033] *A. fumigatus*, *A. terreus* CBS phytase and consensus phytase were overexpressed in *Hansenula polymorpha*. *A. niger* and *A. nidulans* phytase were overexpressed in *A. niger* Cloning, purification and characterization of these phytases was previously described by Pasamontes *et al* [Appl. Environ. Microbiol. (1997), 63, p. 1696-1700]. Construction, cloning and purification of consensus phytase were performed according to European Patent Application Publication No. 897 985. Non-formulated consensus phytase had an increased thermal stability of up to 70 °C and, due to an amino acid exchange (L at position 50 for Q), a three-fold higher specific activity compared to *A. fumigatus* phytase.

#### c) Phytase activity assay

[0034] For the determination of thermostability the enzymatic activity measurements with phytic acid were done at different temperatures by diluting the purified enzymes to 0.05 U/ml (activities measured at 37 °C) in 0.2 M sodium acetate, pH 5.0 (+/- additives in % w/w). An aliquot of the protein solution (250  $\mu$ l) was preincubated for 5 mm at the desired temperature, followed by addition of an equal volume of a solution containing 1% phytic acid in 0.2 M sodium acetate, pH 5.0 (preincubated as a 10 ml aliquot for 10 mm at the same temperature). After incubation of the sample for 15 mm at the desired temperature (e.g. at 60 or 65 °C for the screening of additive effects), the reaction was stopped by addition of 0.5 ml 15% trichloroacetic acid. Determination of liberated inorganic phosphate was performed by standard methods.

#### d) Evaluation of thermostabilizing additives

[0035] In general, the polyols have been dissolved at a concentration of 25 or 50% (w/w) in 0.2 M sodium acetate, pH 5.0. PEGs have been dissolved at a concentration of 50% with the exception of PEGs with a molecular weight of 4000, 8000 and 10000 which were used at a concentration of 25%. For the screening of PEGs and other polyols, the preincubation and reaction temperature was chosen as 60 °C for *A. nidulans* and *A. terreus* CBS phytase, 65 °C for *A. fumigatus* and *A. niger* phytase and 75 °C for consensus phytase.

[0036] Disodium malonate, succinate and glutarate were dissolved at concentrations of 5, 10 and 25% and phytase activity was measured after preincubation of enzyme plus additive and substrate (see above) at the following temperatures: 37, 45, 50, 55, 60, 65, 70, 75, 80, and 85 °C. In the same way, the temperature dependence of the activity of different phytases in the presence of 25% xylitol and ribitol was tested. It should be noted that the concentration of the additives was reduced by half after substrate addition.



## e) Crosslinking of carbohydrate chains

[0037] Crosslinking of phytase carbohydrate chains was performed as described for invertase by Cesi et al. [Studies in Organic Chemistry 47: Stability and Stabilization of Enzymes, Proceedings of an International Symposium held in Maastricht, The Netherlands, 1992, Elsevier Science Publications B.V., Amsterdam, The Netherlands]. Phytase samples (5 mg protein/ml) were incubated for 2 h at 30 °C in the presence of different concentrations (0, 5, 10, 20, 30, 40 and 50 mM) of sodium periodate in 0.2 M sodium acetate, pH 5.0, and stored at 4 °C overnight. Each sample was desalted on a 5-ml HiTrap desalting column (Pharmacia) connected to an ÄktaExplorer system (Pharmacia), using 0.2 M sodium acetate, pH 5.0, as elution buffer. Crosslinking was achieved by adding 100 µl of 0.5 M adipic acid dihydrazide dissolved in 0.2 M sodium acetate, pH 5.0, to 900 µl of the desalted oxidation products. Phytase activity measurements and gel electrophoresis of the samples were performed after both the oxidation and crosslinking steps.

## f) High-shear granulation of thermostabilized phytases

[0038] 100-250 ml of a phytase solution (in total 2500 - 5000 units of crosslinked or non-crosslinked phytase) were added to 1 kg of a dry mixture of 5-10% calcium lignosulfonate (Borregard, Norway), 5-20% silica (Sipernat 50S, Degussa, Germany), 0-20% thermostabilizing agent and gypsum. During the high-shear granulation process water was added until granulates with desired properties were formed. The granulates were dried in a fluid bed dryer for 15 mm at 45 °C and subsequently fat coated with natural palm fat (Palm 46, Florin, Basel, Switzerland).

## g) Pelleting stability of thermostabilized dry and liquid phytase formulations

[0039] Thermostabilized dry or liquid formulations of phytases (as mentioned above) were mixed with feed and subsequently pelleted under steam conditioning at 85 °C. The pelleting stability of phytase was determined by measurement of the phytase activity both in the mash before pelleting and in the delivered pellets.

Example 2

[0040] Investigations of the temperature dependence of activity of different fungal phytases as described in Example 1 revealed activity maxima at the following temperatures: 55 °C for *A. fumigatus* phytase and *A. niger* phytase, 45 °C for *A. terreus* CBS phytase and *A. nidulans* phytase, and 65 °C for consensus phytase. A temperature 10-15 °C above the determined temperature maximum was chosen as screening point for studying the effects of polyols, polyethylene glycols, dicarboxylates, carboxymethylcellulose and sodium alginate on the thermostability of phytases.

## a) Addition of polyethylene glycols of different molecular weights

[0041] The addition of 50% or 25% (25% and 12.5% final concentration during the reaction period) polyethylene glycol enhanced the specific activity of *A. fumigatus* phytase measured at 65 °C in a molecular weight-dependent fashion, with a maximum being observed with PEG 1450 (specific activity 80 U\*(mg protein)<sup>-1</sup>) and considerable activities also with PEG 1000 (50 U\*(mg protein)<sup>-1</sup>) and PEG 3350 (42 U\*(mg protein)<sup>-1</sup>). The results of this experiment are summarized in Figure 2.

[0042] PEGs with molecular weights of 600, 1000, 1450, 3350 and 4000 Da showed similar effects on the other phytases tested. The results of this experiment are shown in Figure 3.

## b) Addition of polyols

[0043] The polyols ribitol, xylitol (C<sub>5</sub> sugars) and sorbitol (C<sub>6</sub> sugar) in concentrations of 25 and 50% significantly improved the thermostability of *A. fumigatus* phytase. This is shown in Figure 4.

[0044] Erythritol, mannitol, mannoheptulose and mannoheptose were not soluble in 0.2 M sodium acetate, pH 5.0, at a concentration of 50% (w/w) and, therefore, only the 25% values are shown. The specific activities measured at 65 °C were 11, 21 and 11 U\*(mg protein)<sup>-1</sup> in the presence of 25% ribitol, xylitol and sorbitol, and 51, 90 and 74 U\*(mg protein)<sup>-1</sup> in the presence of 50% solutions of ribitol, xylitol and sorbitol, respectively.

[0045] Polyols containing more than 6 or less than 5 carbon atoms such as glycerol (C<sub>3</sub> sugar), erythritol (C<sub>4</sub> sugar), mannoheptose and mannoheptulose (C<sub>7</sub> sugars) showed an inferior effect on the thermostabilization of *A. fumigatus* phytase.

[0046] Xylitol at a concentration of 50% also increased the temperature optimum of *A. nidulans*, *A. terreus* CBS, *A. niger* and consensus phytase by 10-15 °C. The results are shown in Figure 5.

## c) Addition of dicarboxylic acids

[0047] Malonate, succinate and glutarate at a concentration of 25% (12.5% final concentration in the activity assay) resulted in a significant increase in *A. fumigatus* phytase thermostability with considerable activity still being detected at 70 °C for malonate and at 65 °C for succinate and glutarate. The results are shown in Figure 6.

[0048] In addition, dicarboxylates stimulated *A. fumigatus* phytase activity measured at 37 °C, with an approximately 4-fold increase in phytase activity in the case of malonate, a 2-fold increase for succinate and minor effects for glutarate. Investigation of different concentrations (5, 10 and 25%) of malonate showed that thermostabilization of *A. fumigatus* phytase is concentration-dependent whereas stimulation of enzymatic activity, at least in this concentration range, is not. This is shown in Figure 7.

[0049] In contrast to these findings, different concentrations of sodium acetate (5, 10 and 25%), a monocarboxylic acid, caused a 2-fold increase in specific activity of *A. fumigatus* phytase at 37 °C, but had only minor effects on the thermal stability of the protein. This can be seen in Figure 8.

[0050] Disodium malonate and succinate generally increased the thermostability of *A. nidulans*, *A. terreus* CBS, *A. niger* and consensus phytase by 5-15 °C. On the other hand, stimulation of phytase activity was only observed for *A. nidulans* and *A. fumigatus* phytase, both having a rather low specific activity, but not for *A. terreus* CBS, *A. niger* and consensus phytase. This is demonstrated in Figures 9 and 10.

## d) Effect of crosslinking

[0051] In a preliminary experiment, *A. fumigatus* phytase monomers were crosslinked by incubation with glutaraldehyde. The resulting thermostabilization measured at 60 °C reached a maximum after 1 hr reaction time but led to activity loss (measured at 37 °C). In a further set of experiments, *A. fumigatus* phytase monomers were crosslinked via their carbohydrate chains. This type of crosslinking was achieved with only minor loss of specific activity (< 10%) and resulted in the formation of oligomeric forms at sodium periodate concentrations above 15 mM. This can be seen from Figure 11.

[0052] The extent of thermostabilization was dependent on periodate concentration and reached a maximum at 50 mM where high specific activities were observed up to 75 °C (see Figure 12). A pronounced effect of phytase oligomerization on thermostability was also observed for consensus phytase crosslinked via its carbohydrate chains. This can be seen from Figure 12.

[0053] In the present work, we focused our efforts on the thermostabilization effects of low-M<sub>r</sub> additives - which are highly recommended for stabilization of industrial enzymes - and of chemical modification - even though this latter approach is commonly regarded as less attractive for technical and economical reasons.

[0054] We have found thermostabilization by C<sub>5</sub> sugars for a range of different phytases expressed in filamentous fungi (*A. niger*) or yeasts (*Hansenula polymorpha*). The increase in thermostability varied to some extent between the different phytases, but was around 10 °C. The effect of PEGs was molecular weight-dependent. The optimal thermostabilization of all phytases was obtained with PEGs having a molecular weight between 1000 and 3350 Da.

[0055] Sodium acetate, a monocarboxylic acid and main component of the standard phytase activity assay, caused a concentration-dependent increase in *A. fumigatus* phytase activity, but had no effect on phytase thermostability. Therefore, carboxylate groups might be responsible for the activity modulation whereas bifunctional dicarboxylates possibly stabilize phytases by ionic interactions.

Example 3

## Design of the amino acid sequence of consensus phytase-1

## Alignment of the amino acid sequences

[0056] The alignment was calculated using the program PILEUP from the Sequence Analysis Package Release 9.0 (Devereux *et al.*, 1984) with the standard parameter (gap creation penalty 12, gap extension penalty 4). The location of the gaps was refined using a text editor. Table 1 shows the sequences (see Figure 13) without the signal sequence that were used for the performance of the alignment starting with the amino acid (aa) as mentioned in Table 1.

Table 1

Origin and vote weight of the phytase amino acid sequences used for the design of consensus phytase-1

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-phyA from *Aspergillus terreus* 9A-1, aa 27, vote weight 0.5 (Mitchell *et al.*, 1997)

Table 1 (continued)

Origin and vote weight of the phytase amino acid sequences used for the design of consensus phytase-1

	-phyA from <i>Aspergillus terreus</i> cbs116.46, aa 27, vote weight 0.5 (van Loon <i>et al.</i> , 1998)
5	- phyA from <i>Aspergillus niger</i> var. <i>awamori</i> , aa 27, vote weight 0.33 (Piddington <i>et al.</i> , 1993)
	-phyA from <i>Aspergillus niger</i> T213, aa 27, vote weight 0.33
	- phyA from <i>Aspergillus niger</i> strain NRRL3135, aa 27, vote weight 0.33 (van Hartingsveldt <i>et al.</i> , 1993)
10	- phyA from <i>Aspergillus fumigatus</i> ATCC 13073, aa 26, vote weight 0.2 (Pasamontes <i>et al.</i> , 1997)
	-phyA from <i>Aspergillus fumigatus</i> ATCC 32722, aa 26, vote weight 0.2 (van Loon <i>et al.</i> , 1998)
	- phyA from <i>Aspergillus fumigatus</i> ATCC 58128, aa 26, vote weight 0.2 (van Loon <i>et al.</i> , 1998)
	- phyA from <i>Aspergillus fumigatus</i> ATCC 26906, aa 26, vote weight 0.2 (van Loon <i>et al.</i> , 1998)
15	- phyA from <i>Aspergillus fumigatus</i> ATCC 32239, aa 30, vote weight 0.2 (van Loon <i>et al.</i> , 1998)
	-phyA from <i>Emericella nidulans</i> , aa 25, vote weight 1.0, Pasamontes <i>et al.</i> , 1997a)
	- phyA from <i>Talaromyces thermophilus</i> ATCC 20186, aa 24, vote weight 1.0 (Pasamontes <i>et al.</i> , 1997a)
20	-phyA from <i>Myceliophthora thermophila</i> , aa 19, vote weight 1.0 (Mitchell <i>et al.</i> , 1997)

#### Calculation of the amino acid sequence of consensus phytase-1

[0057] Using the refined alignment as input, the consensus sequence was calculated by the program PRETTY from the Sequence Analysis Package Release 9.0 (Devereux *et al.*, 1984). PRETTY prints sequences with their columns aligned and can display a consensus sequence for an alignment. A vote weight that pays regard to the similarity between the amino acid sequences of the phytases aligned was assigned to all sequences. The vote weight was set such as the combined impact of all phytases from one sequence subgroup (same species, but from different strains), e. g. the amino acid sequences of all phytases from *A. fumigatus*, on the election was set one, that means that each sequence contributes with a value of 1 divided by the number of strain sequences (see Table 1). By this means, it was possible to prevent that very similar amino acid sequences, e. g. of the phytases from different *A. fumigatus* strains, dominate the calculated consensus sequence.

[0058] The program PRETTY was started with the following parameters: The plurality defining the number of votes below which there is no consensus was set on 2.0. The threshold, which determines the scoring matrix value below which an amino acid residue may not vote for a coalition of residues, was set on 2. PRETTY used the PrettyPep.Cmp consensus scoring matrix for peptides.

[0059] Ten positions of the alignment (position 46, 66, 82, 138, 162, 236, 276, 279, 280, 308; Figure 13), for which the program was not able to determine a consensus residue, were filled by hand according to the following rules: if a most frequent residue existed, this residue was chosen (138, 236, 280); if a prevalent group of similar or phylogenetically equivalent residues occurred, the most frequent or, if not available, one residues of this group was selected (46, 66, 82, 162, 276, 308). If there was either a prevalent residue nor a prevalent group, one of the occurring residues was chosen according to common assumption on their influence on the protein stability (279). Eight other positions (132, 170, 204, 211, 275, 317, 384, 447; Figure 13) were not filled with the amino acid residue selected by the program but normally with amino acids that occur with the same frequency as the residues that were chosen by the program. In most cases, the slight underrating of the three *A. niger* sequences (sum of the vote weights: 0.99) was eliminated by this corrections.

#### Conversion of the consensus phytase-1 amino acid sequence to a DNA sequence

[0060] The first 26 amino acid residues of *A. terreus* cbs116.46 phrase were used as signal peptide and, therefore, fused to the N-terminus of all consensus phrases. For this stretch, we used a special method to calculate the corresponding DNA sequence. Purvis *et al.* (1987) proposed that the incorporation of rare codons in a gene has an influence on the folding efficiency of the protein. Therefore, at least the distribution of rare codons in the signal sequence of *A. terreus* cbs116.46, which was used for the consensus phrase and which is very important for secretion of the protein, but converted into the *S. cerevisiae* codon usage, was transferred into the new signal sequence generated for expression in *S. cerevisiae*. For the remaining parts of the protein, we used the codon frequency table of highly expressed *S. cerevisiae* genes, obtained from the GCG program package, to translate the calculated amino acid sequence into a DNA sequence.

[0061] The resulting sequence of the *fcp* gene is shown in Figure 14.

#### Construction and cloning of the consensus phytase-1 gene

5 [0062] The calculated DNA sequence of consensus phytase-1 (*fcp*) was divided into oligonucleotides of 85 bp, alternately using the sequence of the sense and the anti-sense strand. Every oligonucleotide overlaps 20 bp with its previous and its following oligonucleotide of the opposite strand. The location of all primers, purchased by Microsynth, Balgach (Switzerland) and obtained in a PAGE-purified form, is indicated in Figure 14.

#### 10 PCR-Reactions

[0063] In three PCR reactions, the synthesized oligonucleotides were composed to the entire gene. For the PCR, the High Fidelity Kit from Boehringer Mannheim (Boehringer Mannheim, Mannheim, Germany) and the thermo cycler The Protokol™ from AMS Biotechnology (Europe) Ltd. (Lugano, Switzerland) was used.

15 [0064] Oligonucleotide CP-1 to CP-10 (Mix 1, Figure 14) were mixed to a concentration of 0.2 pMol/μl of each oligonucleotide. A second oligonucleotide mixture (Mix 2) was prepared with CP-9 to CP-22 (0.2 pMol/μl of each oligonucleotide). Additionally, four short primers were used in the PCR reactions:

20 CP-a: *Eco* RI  
5'-TATATGAATTCATGGGCGTGTTCGTC-3'

25 CP-b:  
5'-TGAAAAGTTCATTGAAGGTTTC-3'

30 CP-c:  
5'-TCTTCGAAAGCAGTACAAGTAC-3'

35 CP-e: *Eco* RI  
5'-TATATGAATTCTTAAGCGAAAC-3'

40

PCR reaction α: 10 μl Mix 1 (2.0 pmol of each oligonucleotide)

45 2 μl nucleotides (10 mM each nucleotide)  
2 μl primer CP-a (10 pmol/μl)  
2 μl primer CP-c (10 pmol/μl)  
10,0 μl PCR buffer  
0.75 μl polymerase mixture  
50 73.25 μl H<sub>2</sub>O

PCR reaction b: 10 μl Mix 2 (2.0 pmol of each oligonucleotide)

55 2 μl nucleotides (10 mM each nucleotide)  
2 μl primer CP-b (10 pmol/μl)  
2 μl primer CP-e (10 pmol/μl)  
10,0 μl PCR buffer  
0.75 μl polymerase mixture (2.6 U)

73.25 µl H<sub>2</sub>O

Reaction conditions for PCR reaction a and b:

step 1	2 min - 45°C
step 2	30 sec - 72°C
step 3	30 sec - 94°C
step 4	30 sec - 52°C
step 5	1 min - 72°C

[0065] Step 3 to 5 were repeated 40-times.

[0066] The PCR products (670 and 905 bp) were purified by an agarose gel electrophoresis (0.9% agarose) and a following gel extraction (QIAEX II Gel Extraction Kit, Qiagen, Hilden, Germany). The purified DNA fragments were used for the PCR reaction c.

PCR reaction c: 6 µl PCR product of reaction a (≈ 50 ng)

6 µl PCR product of reaction b (≈ 50 ng)

2 µl primer CP-a (10 pmol/µl)

2 µl primer CP-e (10 pmol/µl)

10.0 µl PCR buffer

0.75 µl polymerase mixture (2.6 U)

73.25 µl H<sub>2</sub>O

Reaction conditions for PCR reaction c:

step 1	2 min - 94°C
step 2	30 sec - 94°C
step 3	30 sec - 55°C
step 4	1 min - 72°C

[0067] Step 2 to 4 were repeated 31-times.

[0068] The resulting PCR product (1.4 kb) was purified as mentioned above, digested with *Eco* RI, and ligated in an *Eco* RI-digested and dephosphorylated pBsk(-)-vector (Stratagene, La Jolla, CA, USA). 1 µl of the ligation mixture was used to transform *E. coli* XL-1 competent cells (Stratagene, La Jolla, CA, USA). All standard procedures were carried out as described by Sambrook *et al.* (1987). The DNA sequence of the constructed consensus phytase gene (*fcp*, Figure 14) was controlled by sequencing as known in the art.

#### Example 4

##### Design of an improved consensus phytase (consensus phytase-10) amino acid sequence

[0069] The alignments used for the design of consensus phytase-10 were calculated using the program PILEUP from the Sequence Analysis Package Release 9.0 (Devereux *et al.*, 1984) with the standard parameter (gap creation penalty 12, gap extension penalty 4). The location of the gaps was refined using a text editor.

[0070] The following sequences were used for the alignment of the *Basidiomycetes* phytases starting with the amino acid (aa) mentioned in Table 2:

Table 2

Origin and vote weight of five *Basidiomycetes* phytases used for the calculation of the corresponding amino acid consensus sequence (basidio)

- *phyA1* from *Paxillus involutus* NN005693, aa 21, vote weight 0.5 (WO 98/28409)
- *phyA2* from *Paxillus involutus* NN005693, aa 21, vote weight 0.5 (WO 98/28409)
- *phyA* from *Trametes pubescens* NN9343, aa 24, vote weight 1.0 (WO 98/28409)
- *phyA* from *Agrocybe pediades* NN009289, aa 19, vote weight 1.0 (WO 98/28409)
- *phyA* from *Peniophora lycii* NN006113, aa 21, vote weight 1.0 (WO 98/28409)

[0071] The alignment is shown in Figure 3.

[0072] In Table 3 the genes, which were used for the performance of the final alignment, are arranged. The first amino acid (aa) of the sequence which is used in the alignment is mentioned behind the organism designation.

Table 3

Origin and vote weight of the phytase sequences used for the design of consensus phytase 10

- *phyA* from *Aspergillus terreus* 9A-1, aa 27, vote weight 0.5 (Mitchell *et al.*, 1997)
- *phyA* from *Aspergillus terreus* cbs116.46, aa 27, vote weight 0.5 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus niger* var. *awamori*, aa 27, vote weight 0.5 (Piddington *et al.*, 1993)
- *phyA* from *Aspergillus niger* strain NRRL3135, aa 27, vote weight 0.5 (van Hartingsveldt *et al.*, 1993)
- *phyA* from *Aspergillus fumigatus* ATCC 13073, aa 26, vote weight 0.2 (Pasamontes *et al.*, 1997)
- *phyA* from *Aspergillus fumigatus* ATCC 32722, aa 26, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus fumigatus* ATCC 58128, aa 26, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus fumigatus* ATCC 26906, aa 26, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Aspergillus fumigatus* ATCC 32239, aa 30, vote weight 0.2 (van Loon *et al.*, 1998)
- *phyA* from *Emericella nidulans*, aa 25, vote weight 1.0, Pasamontes *et al.*, 1997a)
- *phyA* from *Talaromyces thermophilus* ATCC 20186, aa 24, vote weight 1.0 (Pasamontes *et al.*, 1997a)
- *phyA* from *Myceliophthora thermophila*, aa 19, vote weight 1.0 (Mitchell *et al.*, 1997)
- *phyA* from *Thermomyces lanuginosa*, aa 36, vote weight 1.0 (Berka *et al.*, 1998)
- Consensus sequence of five *Basidiomycetes* phytases, vote weight 1.0 (Basidio, Figure 15)

[0073] The corresponding alignment is shown in Figure 16.

#### Calculation of the amino acid sequence of consensus-10

[0074] To improve the alignment, we added the original consensus sequence of five phytases from four different *Basidiomycetes*, called Basidio, still containing the undefined sequence positions (see Figure 15), nearly all phytase sequences used for calculation of the original consensus phytase and one new phytase sequence from the *Ascomycete* *Thermomyces lanuginosa* to a larger alignment. Using the consensus sequence of the basidiomycetal phytase sequences, does not pay regard to the diversity among the five amino acid sequences, but pays regard to the common and different amino acid residues between the phytases from the *Ascomycetes* and the *Basidiomycetes*.

[0075] We set plurality on 2.0 and threshold on 3. The used vote weight are listed in Table 3. The alignment and the corresponding consensus sequence is presented in Figure 16. The new consensus phytase sequence has 32 different amino acids in comparison to the original consensus phytase. Positions for which the program PRETTY was not able to calculate a consensus amino acid residue were filled according to rules mentioned in Example 3. None of the residues suggested by the program was replaced.

[0076] Furthermore, we included all *Basidiomycetes* phytases as single amino acid sequences but assigning a vote weight of 0.2 in the alignment. The corresponding alignment is shown in Figure 18. The calculated consensus amino acid sequence (consensus phytase-11) has the following differences to the sequence of consensus phytase-10. Letter X means that the program was not able to calculate a consensus amino acid; the amino acid in parenthesis corresponds to the amino acid finally included into the consensus phytase-10.

D35X, X(K)69K, X(E)100E, A101R, Q134N, X(K)153N, X(H)190H, X(A)204S, X(E)220D, E222T, V227A, X(R)271R, H287A, X(D)288D, X(K)379K, X(I)389I, E390X, X(E)415E, X(A)416A, X(R)446L, E463A, whereas the numbering is as in Fig. 17.

[0077] We also checked single amino acid replacements suggested by the improved consensus sequences 10 and 11 on their influence on the stability of the original consensus phytase. The approach is described in example 5.

Conversion of consensus phytase-10 amino acid sequence to a DNA sequence

[0078] The first 26 amino acid residues of *A. terreus* cbs116.46 phytase were used as signal peptide and, therefore, fused to the *N*-terminus of consensus phytase-10. The used procedure is further described in Example 3.

[0079] The resulting sequence of the *fcp10* gene is shown in Figure 17.

#### Construction and cloning of the consensus phytase-10 gene (*fcp10*)

[0080] The calculated DNA sequence of *fcp10* was divided into oligonucleotides of 85 bp, alternately using the sequence of the sense and the anti-sense strand. Every oligonucleotide overlaps 20 bp with its previous and its following oligonucleotide of the opposite strand. The location of all primers, purchased by Microsynth, Balgach (Switzerland) and obtained in a PAGE-purified form, is indicated in Figure 17.

#### PCR-Reactions

[0081] In three PCR reactions, the synthesized oligonucleotides were composed to the entire gene. For the PCR, the High Fidelity Kit from Boehringer Mannheim (Boehringer Mannheim, Mannheim, Germany) and the thermo cycler The Protokol™ from AMS Biotechnology (Europe) Ltd. (Lugano, Switzerland) was used. The following oligonucleotides were used in a concentration of 0.2 pMol/μl.

Mix 1.10: CP-1, CP-2, CP-3.10, CP-4.10, CP-5.10, CP-6, CP-7.10, CP-8.10, CP-9.10, CP-10.10

Mix 2.10: CP-9.10, CP-11.10, CP-12.10, CP-13.10, CP-14.10, CP-15.10, CP-16.10, CP-17.10, CP18.10, CP-19.10, CP-20.10, CP-21.10, CP-22.10

[0082] The newly synthesized oligonucleotides are marked by number 10. The phytase contains the following 32 exchanges, which are underlined in Figure 17, in comparison to the original consensus phytase: Y54F, E58A, D69K, D70G, A94K, N134Q, I158V, S187A, Q188N, D197N, S204A, T214L, D220E, L234V, A238P, D246H, T251N, Y259N, E267D, E277Q, A283D, R291I, A320V, R329H, S364T, I366V, A379K, S396A, G404A, Q415E, A437G, A463E.

[0083] Four short PCR primer were used for the assembling of the oligonucleotides:

CP-a: *Eco* RI  
5'-TATATGAATTTCATGGGCGTGTTTCGTC-3'

CP-b:  
5'-TGAAAAGTTCATTGAAGGTTTC-3'

CP-c.10:  
5'-TCTTCGAAAGCAGTACACAAAC-3'

CP-e: *Eco* RI  
5'-TATATGAATTCTTAAGCGAAAC-3'

PCR reaction a: 10 µl Mix 1.10 (2.0 pmol of each oligonucleotide)

2 µl nucleotides (10 mM each nucleotide)  
2 µl primer CP-a (10 pmol/ml)  
2 µl primer CP-c.10 (10 pmol/ml)  
10,0 µl PCR buffer  
0.75 µl polymerase mixture  
73.25 µl H<sub>2</sub>O

PCR reaction b: 10 µl Mix 2.10 (2.0 pmol of each oligonucleotide)

2 µl nucleotides (10 mM each nucleotide)  
2 µl primer CP-b (10 pmol/ml)  
2 µl primer CP-e (10 pmol/ml)  
10,0 µl PCR buffer  
0.75 µl polymerase mixture (2.6 U)  
73.25 µl H<sub>2</sub>O

Reaction conditions for PCR reaction a and b:

step 1	2 min - 45°C
step 2	30 sec - 72 °C
step 3	30 sec - 94 °C
step 4	30 sec - 52 °C
step 5	1 min - 72°C

[0084] Step 3 to 5 were repeated 40-times.

[0085] The PCR products (670 and 905 bp) were purified by an agarose gel electrophoresis (0.9% agarose) and a following gel extraction (QIAEX II Gel Extraction Kit, Qiagen, Hilden, Germany). The purified DNA fragments were used for the PCR reaction c.



PCR reaction c: 6 µl PCR product of reaction a ≈50 ng)

6 µl PCR product of reaction b ≈50 ng)

2 µl primer CP-a (10 pmol/ml)

5 2 µl primer CP-e (10 pmol/ml)

10,0 µl PCR buffer

0.75 µl polymerase mixture (2.6 U) 73.25 µl H<sub>2</sub>O

Reaction conditions for PCR reaction c:

10

step 1	2 min - 94°C
step 2	30 sec - 94 °C
step 3	30 sec - 55 °C
step 4	1 min - 72 °C

15

20 [0086] Step 2 to 4 were repeated 31-times.

[0087] The resulting PCR product (1.4 kb) was purified as mentioned above, digested with *Eco* RI, and ligated in an *Eco* RI-digested and dephosphorylated pBsk(-)-vector (Stratagene, La Jolla, CA, USA). 1 µl of the ligation mixture was used to transform *E. coli* XL-1 competent cells (Stratagene, La Jolla, CA, USA). All standard procedures were carried out as described by Sambrook *et al.* (1987). The DNA sequence of the constructed gene (*fcp10*) was checked by sequencing as known in the art.

25

#### Example 5

30 Increasing the thermostability of consensus phytase-1 by introduction of single mutations suggested by the amino acid sequence of consensus phytase-10 and consensus phytase-11

[0088] In order to increase the thermostability of homologous genes, it is also possible to test the stability effect of each differing amino acid residue between the protein of interest and the calculated consensus sequence and to combine all stabilizing mutations into the protein of interest. We used the consensus phytase as protein of interest and tested the effect on the protein stability of 34 amino acid residues, differing to consensus phytase 10 and/or 11 as single mutations.

35

[0089] To construct muteins for expression in *A. niger*, *S. cerevisiae*, or *H. polymorpha*, the corresponding expression plasmid containing the consensus phytase gene was used as template for site-directed mutagenesis (see Example 8 - 10). Mutations were introduced using the "quick exchange"™ site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA) following the manufacturer's protocol and using the corresponding primers. All mutations made and their corresponding primers are summarized in Table 4. Plasmids harboring the desired mutation were identified by DNA sequence analysis as known in the art.

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Table 4: Primers used for site-directed mutagenesis of consensus phytase

5 (Exchanged bases are highlighted in bold. The introduction of a restriction site is marked above the sequence. When a restriction site is written in parenthesis, the mentioned site was destroyed by introduction of the mutation.)

10

mutation      Primer set

15

*Kpn I*-

Q50T      5'-CACTTGTGGGGT**A**CCTACTCTCCATACTTCTC-3'  
20      5'-GAGAAGTATGGAGAGTAG**GT**ACCCCAAGTG-3'

25

Y54F      5'-GGTCAATACTCTCCATTCTTCTCTTTGGAAG-3'  
5'-CTTCCAAAGAGAAGAATGGAGAGTATTGACC-3'

30

E58A      5'-CATACTTCTCTTTGGCAGACGAATCTGC-3'  
5'-GCAGATTCGTCTGCCAAAGAGAAGTATG-3'

35

*Aat II*

D69K      5'-CTCCAGACGTCCCAAAGGACTGTAGAGTTAC-3'  
40      5'-GTAACCTCTACAGTCCTTTGGG**G**ACGTCTGGAG-3'

40

*Aat II*

D70G      5'-CTCCAGACGTCCCAAGACGGCTGTAGAGTTAC-3'  
45      5'-GTAACCTCTACAGCCGTCTGGG**G**ACGTCTGGAG-3'

45

50

K91A      5'-GATACCCAACCTTCTTCTGCGTCTAAGGCTTACTCTG-3'  
5'-CAGAGTAAGCCTTAGACGCAGAAGAAGTTGGGTATC-3'

55

*ScaI*

A94K 5'-CTTCTAAGTCTAAGAAGTACTCTGCTTTG-3'  
5'-CAAAGCAGAGTACTTCTTAGACTTAGAAG-3'

A101R 5'-GCTTACTCTGCTTTGATTGAACGGATTCAAAAGAACGCTAC-3'  
5'-GTAGCGTTCTTTTGAATCCGTTCAATCAAAGCAGAGTAAGC-3'

N134Q 5'-CCATTCGGTGAACAGCAAATGGTTAACTC-3'  
5'-GAGTTAACCATTTGCTGTTCAACCGAATGG-3'

*Nru I*

K153N 5'-GATACAAGGCTCTCGCGAGAAACATTGTTC-3'  
5'-GGAACAATGTTTCTCGCGAGAGCCTTGTATC-3'

*Bss HI*

I158V 5'-GATTGTTCCATTTCGTGCGCGCTTCTGGTTC-3'  
5'-GAACCAGAAGCGCGCACGAATGGAACAATC-3'

*Bcl I*

D197N 5'-CTCCAGTTATTAACGTGATCATTCCAGAAGG-3'  
5'-CCTTCTGGAATGATCACGTTAATAACTGGAG-3'

*Apa I*

S187A 5'-GGCTGACCCAGGGGCCCAACCACACCAAGC-3'  
5'-GCTTGGTGTGGTTGGGCCCCTGGGTCAGCC-3'

*Nco I*

T214L 5'-CACTTTGGACCATGGTCTTTGTACTGCTTTCG-3'  
5'-CGAAAGCAGTACAAAGACCATGGTCCAAAGTG-3'

*Avr II*

E222T 5'-GCTTTCGAAGACTCTACCTAGGTGACGACGTTG-3'  
5'-CAACGTCGTCACCTAGGGTAGAGTCTTCGAAAGC-3'

5  
 V227A            5'-GGTGACGACGCTGAAGCTAACTTCAC-3'  
                   5'-GTGAAGTTAGCTTCAGCGTCGTCACC-3'

*Sac* II

10  
 L234V            5'-CTAACTTCACCGCGGTGTTGCTCCAG-3'  
                   5'-CTGGAGCGAACACCGCGGTGAAGTTAG-3'

15  
 A238P           5'-GCTTTGTTGCTCCACCTATTAGAGCTAGATTGG-3'  
                   5'-CCAATCTAGCTCTAATAGGTGGAGCGAACAAAGC-3'

*Hpa* I

20  
 T251N           5'-GCCAGGTGTTAACTTGACTGACGAAG-3'  
                   5'-TTCGTCAGTCAAGTTAACACCTGGC-3'

*Aat* II

25  
 Y259N           5'-GACGAAGACGTCGTTAACTTGATGGAC-3'  
                   5'-GTCCATCAAGTTAACGACGTCCTTCGTC-3'

30  
                   *Asp* I

35  
 E267D           5'-GTCCATTCGACACTGTCGCTAGAACTT C-3'  
                   5'-GAAGTTCTAGCGACAGTGTCGAATGGAC-3'

40  
 E277Q           5'-CTGACGCTACTCAGCTGTCTCCATT C-3'  
                   5'-GAATGGAGACAGCTGAGTAGCGTCAG-3'

45  
 A283D           5'-GTCTCCATTCTGTGATTTGTTCACTCAC-3'  
                   5'-GTGAGTGAACAAATCACAGAATGGAGAC-3'

*Ksp* I

50  
 H287A           5'-GCTTTGTTACCGCGGACGAATGGAG-3'  
                   5'-CTCCATTCTGTCGCGGTGAACAAAGC-3'

55

*Bam* HI

5 R291I 5'-CACGACGAATGGATCCAATACGACTAC-3'  
5'-GTAGTCGTATTGGATCCATTCGTCGTG-3'

*Bsi* WI

10 Q292A 5'-GACGAATGGAGAGCGTACGACTACTTG-3'  
5'-CAAGTAGTCGTACGCTCTCCATTCGTC-3'

*Hpa* I

15 A320V 5'-GGTGTGTTGGTTTCGTTAACGAATTGATTGC-3'  
5'-GCAATCAATTCGTTAACGAAACCAACACC-3'

## (Bgl II)

20 R329H 5'-GCTAGATTGACTCACTCTCCAGTTCAAG-3'  
25 5'-CTTGA~~A~~CTGGAGAGTGAGTCAATCTAGC-3'

*Eco* RV

30 S364T 5'-CTCACGACAACACTATGATATCTATTTTCTTC-3'  
5'-GAAGAAAATAGATATCATAGTGTTGTCGTGAG-3'

*Nco* I

35 I366V 5'-CGACA~~A~~CTCCATGGTTTCTATTTTCTTCGC-3'  
5'-GCGAAGAAAATAGAAACCATGGAGTTGTTCG-3'

*Kpn* I

40 A379K 5'-GTACAACGGTACCAAGCCATTGTCTAC-3'  
5'-GTAGACAATGGCTTGGTACCGTTGTAC-3'

45

S396A 5'-CTGACGGTTACGCTGCTTCTTGGAC-3'  
50 5'-GTCCAAGAAGCAGCGTAACCGTCAG-3'

55

G404A      5'-CTGTTCCATTCGCTGCTAGAGCTTAC-3'  
               5'-GTAAGCTCTAGCAGCGAATGGAACAG-3'

5

Q415E      5'-GATGCAATGTGAAGCTGAAAAGGAACC-3'  
               5'-GGTTCCTTTTCAGCTTCACATTGCATC-3'

10

*Sal I*

A437G      5'-CACGGTTGTGGTGTGACAAGTTGGG-3'  
               5'-CCCAACTTGTCGACACCACAACCGTG-3'

15

*Mun I*

A463E      5'-GATCTGGTGGCAATTGGGAGGAATGTTTCG-3'  
               5'-CGAAACATTCTCCCAATTGCCACCAGATC-3'

20

25

and accordingly for other mutations.

30 **[0090]** The temperature optimum of the purified phytases, expressed in *Saccharomyces cerevisiae* (Example 9), was determined as outlined in Example 11. Table 5 shows the effect on the stability of consensus phytase for each mutation introduced.

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Table 5

Stability effect of the individual amino acid replacements in consensus phytase-1					
(+ or - means a positive, respectively, negative effect on the protein stability up to 1 °C, ++ and -- means a positive, respectively, negative effect on the protein stability between 1 and 3 °C; the number 10 or 11 corresponds to the consensus phytase sequence that suggests the amino acid replacement.)					
stabilizing		neutral		destabilizing	
mutation	effect	mutation	effect	mutation	effect
E58A (10)	+	D69A	±	Y54F (10)	-
D69K (11)	+	D70G (10)	±	V73I	-
D197N (10)	+	N134Q (10)	±	A94K (10)	-
T214L (10)	++	G186H	±	A101R (11)	-
E222T (11)	++	S187A (10)	±	K153N (11)	-
E267D (10)	+	T214V	±	I158V (10)	--
R291I*	+	T251N (10)	±	G203A	--
R329H (10)	+	Y259N (10)	±	G205S	-
S364T (10)	++	A283D (10)	±	A217V	-
A379K (11)	+	A320V (10)	±	V227A (11)	--
G404A (10)	++	K445T	±	L234V (10)	-
		A463E (10)	±	A238P (10)	--
				E277Q (10)	-
				H287A (11)	-
				Q292A (10)	-
				I366V (10)	-
				S396A (10)	--
				Q415E (11)	-
				A437G (10)	--
				E451R	--

\*: This amino acid replacement was found in another round of mutations.

[0091] We combined eight positive mutations (E58A, D197N, E267D, R291I, R329H, S364T, A379K, G404A) in the consensus phytase using the primers and the technique mentioned above in this example. Furthermore, the mutations Q50T and K91A were introduced which mainly influence the catalytical characteristics of the phytase (see European Patent Application Publication No. 897 985 as well as Example 11). The DNA and amino acid sequence of the resulting phytase gene (consensus phytase-thermo[8]-Q50T-K91A) is shown in Figure 19. In this way, the temperature optimum and the melting point of the consensus phytase was increased by 7 °C (Figure 27, 28, 29).

[0092] Using the results of Table 5, we further improved the thermostability of consensus phytase 10 by the following back mutations K94A, V158I, and A396S that revealed a strong negative influence on the stability of consensus phytase. The resulting protein is phytase-10-thermo [3]. Furthermore, we introduced the mutations Q50T and K91A which mainly influence the catalytical characteristics of consensus phytase (see patent application EP Publication No. 897 985 as well as Example 11 and Figure 26 and 27). The resulting DNA and amino acid sequence is shown in Figure 20. The optimized phytase showed a 4 °C higher temperature optimum and melting point than consensus phytase 10 (Figure 24 and 25). Furthermore, the phytase has also a strongly increased specific activity with phytate as substrate of 250 U/mg at pH 5.5 (Figure 26).

**Example 6****Stabilization of the phytase of *A. fumigatus* ATCC 13073 by replacement of amino acid residues with the corresponding consensus phytase-1 and consensus phytase-10 residues**

5 [0093] At six typical positions where the *A. fumigatus* 13073 is the only or nearly the only phytase in the alignment of Figure 13 that does not contain the corresponding consensus phytase amino acid residue, the non-consensus amino acid residue was replaced by the consensus one. In a first round, the following amino acids were substituted in *A. fumigatus* 13073 phytase, containing the Q27T substitution and the signal sequence of *A. terreus* cbs.116.46 phytase (see Figure 21):

F55(28)Y, V100(73)I, F114(87)Y, A243(220)L, S265(242)P, N294(282)D.

[0094] The numbers in parentheses confer to the numbering of Figure 13.

15 [0095] In a second round, four of the seven stabilizing amino acid exchanges (E59A, R329H, S364T, G404A) found in the consensus phytase-10 sequence and, tested as single mutation in consensus phytase-1 (Table 5), were additionally introduced into the *A. fumigatus*  $\alpha$ -mutant. Furthermore, the amino acid replacement S126N, shown to reduce the protease susceptibility of the phytase, was introduced.

20 [0096] The mutations were introduced as described in example 5 (see Table 6) and expressed as described in example 8 to 10. The resulting *A. fumigatus* 13073 phytase variants were called  $\alpha$ -mutant and  $\alpha$ -mutant-E59A-S126N-R329H-S364T-G404A.

25 [0097] The temperature optimum (60 °C, Figure 32) and the melting point (67.0 °C, Figure 31) of the *A. fumigatus* 13073 phytase  $\alpha$ -mutant was increased by 5 °C in comparison to the values of the wild-type (temperature optimum: 55 °C,  $T_m$ : 60 °C). The five additional amino acid replacements further increased the temperature optimum by 3 °C (Figure 32).



Table 6: Mutagenesis primers for stabilization of *A. fumigatus* phytase ATCC 13073

5	Mutation	Primer
	F55Y	5'-CACGTACTCGCCATACTTTTCGCTCGAG-3' 5'-CTCGAGCGAAAAGTATGGCGAGTACGTG-3'
10		( <i>Xho</i> I)
	E58A	5'-CCATACTTTTCGCTCGCGGACGAGCTGTCCGTG-3' 5'-CACGGACAGCTCGTCCGCGAGCGAAAAGTAGG-3'
15		
	V100I	5'-GTATAAGAAGCTTATTACGGCGATCCAGGCC-3' 5'-GGCCTGGATCGCCGTAATAAGCTTCTTATAC-3'
20		
	F114Y	5'-CTTCAAGGGCAAGTACGCCTTTTGAAGACG-3' 5'-CGTCTTCAAAAAGGCGTACTTGCCCTTGAAG-3'
25		
	A243L	5'-CATCCGAGCTCGCCTCGAGAAGCATCTTC-3' 5'-GAAGATGCTTCTCGAGGCGAGCTCGGATG-3'
30		
	S265P	5'-CTAATGGATGTGTCCGTTTGATACGGTAG-3' 5'-CTACCGTATCAAACGGACACATGTCCATTAG-3'
35		
	N294D	5'-GTGGAAGAAGTACGACTACCTTCAGTC-3' 5'-GACTGAAGGTAGTCGTACTTCTTCCAC-3'
40		
		( <i>Mlu</i> I)
	R329H	5'-GCCCCGGTTGACGCAATTCGCCAGTGCAGG-3' 5'-CCTGCACTGGCGAATGCGTCAACCGGGC-3'
45		
50		
55		

*Nco* I

S364T 5'-CACACGACAACACCATGGTTTCCATCTTC-3'  
 5'-GAAGATGGAAACCATGGTGTGTCGTGTG-3'

*(Bss* HI)

G404A 5'-GTGGTGCCTTTCGCCGCGGAGCCTACTTC-3'  
 5'-GAAGTAGGCTCGCGCGGCGAAAGGCACCAC-3'

**Example 7****20** Introduction of the active site amino acid residues of the *A. niger* NRRL 3135 phytase into the consensus phytase-1

[0098] We used the crystal structure of the *Aspergillus niger* NRRL 3135 phytase to define all active site amino acid residues (see Reference Example and EP 897 010). Using the alignment of Figure 13, we replaced the following active site residues and additionally the not identical adjacent ones of the consensus phytase by that of the *A. niger* phytase:

S89D, S92G, A94K, D164S, P201S, G203A, G205S, H212P, G224A, D226T, E255T, D256E, V258T, P265S, Q292H, G300K, Y305H, A314T, S364G, M365I, A397S, S398A, G404A, and A405S

[0099] The new protein sequence consensus phytase -7 was backtranslated into a DNA sequence (Figure 22) as described in Example 3. The corresponding gene (*fcp7*) was generated as described in Example 3 using the following oligonucleotide mixes:

Mix 1.7: CP-1, CP-2, CP-3, CP-4.7, CP-5.7, CP-6, CP-7, CP-8.7, CP-9, CP-10.7

Mix 2.7: CP-9, CP-10.7, CP-11.7, CP-12.7, CP-13.7, CP-14.7, CP-15.7, CP-16, CP-17.7, CP-18.7, CP-19.7, CP-20, CR-21, CP-22.

[0100] The DNA sequences of the oligonucleotides are indicated in Figure 15. The newly synthesized oligonucleotides are additionally marked by number 7. After assembling of the oligonucleotides using the same PCR primers as mentioned in Example 3, the gene was cloned into an expression vector as described in Examples 8 - 10.

[0101] The pH-profile determined after expression in *H. polymorpha* and purification was shifted into the acidic range of the pH-spectrum showing an optimum at pH 4.5-5.0 (see Figure 30). The enzyme had a broad pH-optimum reaching at least 60% of its maximum activity from pH 2.5 to pH 6.0. Up to pH 5.0, the profile resembled the profile of the *A. niger* NRRL 3135 phytase. However, below pH 5.0 it lacked the typical low at pH 4.0 of the profile of *A. niger* phytase.

**Example 8**Expression of the consensus phytase genes in *Hansenula polymorpha*

[0102] The phytase expression vectors, used to transform *H. polymorpha* RB11 (Gellissen *et al.*, 1994), was constructed by inserting the *Eco* RI fragment of pBsk*fcp* or variants thereof into the multiple cloning site of the *H. polymorpha* expression vector pFPMT121, which is based on an *ura3* selection marker from *S. cerevisiae*, a formate dehydrogenase (*FMD*) promoter element and a methanol oxidase (*MO*) terminator element from *H. polymorpha*. The 5' end of the *fcp* gene is fused to the *FMD* promoter, the 3' end to the *MOX* terminator (Gellissen *et al.*, 1996; EP 0299 108 B). The resulting expression vector are designated pFPMT*fcp*, pFPMT*fcp*10, pFPMT*fcp*7.

[0103] The constructed plasmids were propagated in *E. coli*. Plasmid DNA was purified using standard state of the art procedures. The expression plasmids were transformed into the *H. polymorpha* strain RP11 deficient in orotidine-5'-phosphate decarboxylase (*ura3*) using the procedure for preparation of competent cells and for transformation of

yeast as described in Gelissen *et al.* (1996). Each transformation mixture was plated on YNB (0.14% w/v Difco YNB and 0.5% ammonium sulfate) containing 2% glucose and 1.8% agar and incubated at 37 °C. After 4 to 5 days individual transformant colonies were picked and grown in the liquid medium described above for 2 days at 37 °C. Subsequently, an aliquot of this culture was used to inoculate fresh vials with YNB-medium containing 2% glucose. After seven further passages in selective medium, the expression vector integrates into the yeast genome in multimeric form. Subsequently, mitotically stable transformants were obtained by two additional cultivation steps in 3 ml non-selective liquid medium (YPD, 2% glucose, 10 g yeast extract, and 20 g peptone). In order to obtain genetically homogeneous recombinant strains an aliquot from the last stabilization culture was plated on a selective plate. Single colonies were isolated for analysis of phytase expression in YNB containing 2% glycerol instead of glucose to derepress the *fmd* promoter. Purification of the consensus phytases was done as described in Example 9.

#### Example 9

##### Expression of the consensus phytase genes in *Saccharomyces cerevisiae* and purification of the phytases from culture supernatant

[0104] The consensus phytase genes were isolated from the corresponding Bluescript-plasmid (pBsk<sup>+</sup>*fcp*, pBSK<sup>+</sup>*fcp10*, pBsk<sup>+</sup>*fcp7*) and ligated into the *Eco* RI sites of the expression cassette of the *Saccharomyces cerevisiae* expression vector pYES2 (Invitrogen, San Diego, CA, USA) or subcloned between the shortened GAPFL (glyceraldehyde-3-phosphate dehydrogenase) promoter and the *pho5* terminator as described by Janes *et al.* (1990). The correct orientation of the gene was checked by PCR. Transformation of *S. cerevisiae* strains, e. g. INVSc1 (Invitrogen, San Diego, CA, USA) was done according to Hinnen *et al.* (1978). Single colonies harboring the phytase gene under the control of the GAPFL promoter were picked and cultivated in 5 ml selection medium (SD-uracil, Sherman *et al.*, 1986) at 30°C under vigorous shaking (250 rpm) for one day. The preculture was then added to 500 ml YPD medium (Sherman *et al.*, 1986) and grown under the same conditions. Induction of the *gal1* promoter was done according to manufacturer's instruction. After four days of incubation cell broth was centrifuged (7000 rpm, GS3 rotor, 15 mm, 5°C) to remove the cells and the supernatant was concentrated by way of ultrafiltration in Amicon 8400 cells (PM30 membranes) and ultra-free-15 centrifugal filter devices (Biomax-30K, Millipore, Bedford, MA, USA). The concentrate (10 ml) was desalted on a 40 ml Sephadex G25 Superfine column (Pharmacia Biotech, Freiburg, Germany), with 10 mM sodium acetate, pH 5.0, serving as elution buffer. The desalted sample was brought to 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and directly loaded onto a 1 ml Butyl Sepharose 4 Fast Flow hydrophobic interaction chromatography column (Pharmacia Biotech, Freiburg, Germany) which was eluted with a linear gradient from 2 M to 0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in 10 mM sodium acetate, pH 5.0. Phytase was eluted in the break-through, concentrated and loaded on a 120 ml Sephacryl S-300 gel permeation chromatography column (Pharmacia Biotech, Freiburg, Germany). Consensus phytase and consensus phytase -7 eluted as a homogeneous symmetrical peak and was shown by SDS-PAGE to be approx. 95% pure.

#### Example 10

##### Expression of the consensus phytase genes in *Aspergillus niger*

[0105] The Bluescript-plasmids pBsk<sup>+</sup>*fcp*, pBSK<sup>+</sup>*fcp10*, and pBsk<sup>+</sup>*fcp7* were used as template for the introduction of a *Bsp* HI-site upstream of the start codon of the genes and an *Eco* RV-site downstream of the stop codon. The Expand™ High Fidelity PCR Kit (Boehringer Mannheim, Mannheim, Germany) was used with the following primers:

Primer Asp-1:

5

*Bsp* HI

5'-TATATCATGAGCGTGTTCGTCGTGCTACTGTTC-3'

10

Primer Asp-2 used for cloning of *fcp* and *fcp7*:

*Eco* RV

3'-ACCCGACTTACAAAGCGAATTCTATAGATATAT-5'

15

Primer Asp-3 used for cloning of *fcp10*:

*Eco* RV

20

3'-ACCCTTCTTACAAAGCGAATTCTATAGATATAT-5'

25 [0106] The reaction was performed as described by the supplier. The PCR-amplified *fcp*-genes had a new *Bsp* HI site at the start codon, introduced by primer Asp-1, which resulted in a replacement of the second amino acid residue glycine by serine. Subsequently, the DNA-fragment was digested with *Bsp* HI and *Eco* RV and ligated into the *Nco* I site downstream of the glucoamylase promoter of *Aspergillus niger* (*glaA*) and the *Eco* RV site upstream of the *Aspergillus nidulans* tryptophan C terminator (*trpC*) (Mullaney *et al.*, 1985). After this cloning step, the genes were sequenced to detect possible failures introduced by PCR. The resulting expression plasmids which basically corresponds to the pGLAC vector as described in Example 9 of EP 684 313, contained the orotidine-5'-phosphate decarboxylase gene (*pyr4*) of *Neurospora crassa* as a selection marker. Transformation of *Aspergillus niger* and expression of the consensus phytase genes was done as described in EP 684 313. The consensus phytases were purified as described in Example 9.

35

#### Example 11

##### Determination of phytase activity and of temperature optimum

40 [0107] Phytase activity was determined basically as described by Mitchell *et al* (1997). The activity was measured in an assay mixture containing 0.5% phytic acid ( $\approx 5$  mM) in 200 mM sodium acetate, pH 5.0. After 15 min of incubation at 37 °C, the reaction was stopped by addition of an equal volume of 15% trichloroacetic acid. The liberated phosphate was quantified by mixing 100  $\mu$ l of the assay mixture with 900  $\mu$ l H<sub>2</sub>O and 1 ml of 0.6 M H<sub>2</sub>SO<sub>4</sub>, 2% ascorbic acid and 0.5% ammonium molybdate. Standard solutions of potassium phosphate were used as reference. One unit of enzyme activity was defined as the amount of enzyme that releases 1  $\mu$ mol phosphate per minute at 37 °C. The protein concentration was determined using the enzyme extinction coefficient at 280 nm calculated according to Pace *et al* (1995): consensus phytase, 1.101; consensus phytase 7, 1.068; consensus phytase 10, 1.039.

50 [0108] In case of pH-optimum curves, purified enzymes were diluted in 10 mM sodium acetate, pH 5.0. Incubations were started by mixing aliquots of the diluted protein with an equal volume of 1% phytic acid ( $\approx 10$  mM) in a series of different buffers: 0.4 M glycine/HCl, pH 2.5; 0.4 M acetate/NaOH, pH 3.0, 3.5; 4.0, 4.5, 5.0, 5.5; 0.4 M imidazole/HCl, pH 6.0, 6.5; 0.4 M Tris/HCl pH 7.0, 7.5, 8.0, 8.5, 9.0. Control experiments showed that pH was only slightly affected by the mixing step. Incubations were performed for 15 min at 37 °C as described above.

55 [0109] For determinations of the substrate specificities of the phytases, phytic acid in the assay mixture was replaced by 5 mM concentrations of the respective phosphate compounds. The activity tests were performed as described above.

[0110] For determination of the temperature optimum, enzyme (100  $\mu$ l) and substrate solution (100  $\mu$ l) were pre-incubated for 5 min at the given temperature. The reaction was started by addition of the substrate solution to the enzyme. After 15 min incubation, the reaction was stopped with trichloroacetic acid and the amount of phosphate released was

determined.

[0111] The pH-optimum of the original consensus phytase was around pH 6.0-6.5 (70 U/mg). By introduction of the Q50T mutation, the pH-optimum shifted to pH 6.0 (130 U/mg). After introduction of K91A, the pH optimum shifted one pH-unit into the acidic pH-range showing a higher specific activity between pH 2.5 and pH 6.0. That was shown for the stabilized mutants and for consensus phytase-10, too (Figure 26 and 27).

[0112] Consensus phytase-7, which was constructed to transfer the catalytic characteristics of the *A. niger* phytase NRRL 3135 into the consensus phytase, had a pH-profile which is shifted into the acidic range of the pH-spectrum showing an optimum between pH 4.5 and 5.0 (see Figure 31). The enzyme had a broad pH-optimum reaching at least 60% of its increased maximum activity from pH 2.5 to pH 6.0. The substrate spectrum, too, resemble more to that of the *A. niger* NRRL 3135 phytase than to the consensus phytase-1.

[0113] The temperature optimum of consensus phytase-1 (71 °C) was 16-26 °C higher than the temperature optimum of the wild-type phytases (45-55 °C, Table 7) which were used to calculate the consensus sequence. The improved consensus phytase-10 showed a further increase of its temperature optimum to 80 °C (Figure 33). The temperature optimum of the consensus phytase-1-thermo[8] was found in the same range (78 °C) using the supernatant of an overproducing *S. cerevisiae* strain. The highest temperature optimum reached of 82 °C was determined for consensus phytase-10-thermo-Q50T-K91A.

Table 7

Temperature optimum and $T_m$ -value of consensus phytase and of the phytases from <i>A. fumigatus</i> , <i>A. niger</i> , <i>E. nidulans</i> , and <i>M. thermophila</i> . The determination of the temperature optimum was performed as described in Example 11. The $T_m$ -values were determined by differential scanning calorimetry as described in Example 12.		
phytase	temperature optimum [°C]	$T_m$ [°C]
Consensus phytase-10-thermo-Q50T-K91A	82	89.3
Consensus phytase-10-thermo-Q50T	82	88.6
Consensus phytase-10	80	85.4
Consensus phytase-1-thermo[8]-Q50T	78	84.7
Consensus phytase-1-thermo[8]-Q50T-K91A	78	85.7
Consensus phytase-1	71	78.1
<i>A. niger</i> NRRL3135	55	63.3
<i>A. fumigatus</i> 13073	55	62.5
<i>A. fumigatus</i> 13073 $\alpha$ -mutant	60	67.0
<i>A. fumigatus</i> 13073 $\alpha$ -mutant (optimized)	63	-
<i>A. terreus</i> 9A-1	49	57.5
<i>A. terreus</i> cbs.116.46	45	58.5
<i>E. nidulans</i>	45	55.7
<i>M. thermophila</i>	55	n. d.
<i>T. thermophilus</i>	45	n. d.

#### Example 12

##### Determination of the melting point by differential scanning calorimetry (DSC)

[0114] In order to determine the unfolding temperature of the phytases, differential scanning calorimetry was applied as previously published by Brugger et al (1997). Solutions of 50-60 mg/ml homogeneous phytase were used for the

tests. A constant heating rate of 10 °C/min was applied up to 90-95 °C.

[0115] The determined melting points reflect the results obtained for the temperature optimums (Table 7). The most stable consensus phytase designed is consensus phytase-10-thermo-Q50T-K91A showing a melting temperature under the chosen condition of 89.3 °C. This is 26 to 33.6 °C higher than the melting point of the wild-type phytases used.

### Example 13

#### Transfer of basidiomycete phytase active site into consensus phytase-10-thermo-Q50T-K91A

[0116] As described previously (Example 5), mutations derived from the basidiomycete phytase active site were introduced into the consensus phytase 10. The following five constructs a) to e) were prepared:

[0117] This construct is called consensus phytase 12, and it comprises a selected number of active site residues of the basidio consensus sequence, its amino acid sequence (consphy12) is shown in Fig. 33 (the first 26 amino acids forms the signal peptide, amended positions are underlined);

a cluster of mutations (Cluster II) was transferred to the consensus 10 sequence, viz.: S80Q, Y86F, S90G, K91A, S92A, K93T, A94R, Y95I;

analogously, another cluster of mutations (Cluster III) was transferred, viz.: T129V, E133A, Q143N, M136S, V137S, N138Q, S139A;

analogously, a further cluster of mutations (Cluster IV) was transferred, viz.: A168D, E171T, K172N, F173W;

and finally, a further cluster of mutations (Cluster V) was transferred, viz.: Q297G, S298D, G300D, Y305T.

[0118] These constructs were expressed as described in Examples 8 to 10.

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[0119]

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## Claims

1. A stabilized dry or liquid enzyme formulation comprising phytase and one or more stabilizing agents selected from the group consisting of:
  - a) C<sub>5</sub> sugars, preferably xylitol or ribitol,
  - b) polyethylene glycols having a molecular weight of 600 to 4000 Da, preferably 1000 to 3350 Da.
  - c) the disodium salts of malonic, glutaric and succinic acid,
  - d) carboxymethylcellulose, and
  - e) alginate, preferably sodium alginate.
2. A stabilized dry or liquid enzyme formulation comprising phytase which has been crosslinked:
  - a) with glutaraldehyde, or by
  - b) oxidation with sodium periodate and reaction with adipic acid dihydrazide.
3. Enzyme formulation according to claims 1 or 2, characterized in that the phytase is a fungal or a consensus phytase.



4. Enzyme formulation according to claim 3, characterized in that the fungal phytase is selected from the group consisting of *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus terreus* and *Aspergillus niger* phytase.
5. Enzyme formulation according to anyone of claims 1 to 4 characterized in that the formulation is liquid.
6. Enzyme formulation according to claim 5, characterized in that the stabilizing agent is polyethylene glycol whereby the polyethylene glycol is present in a concentration of 10-50% (w/w) in the final formulation.
7. Enzyme formulation according to claim 5 or 6, characterized in that the stabilizing agent is xylitol and/or ribitol which is present in the final formulation in a concentration of 20-60% (w/w).
8. Enzyme formulation according to any of claims 5 to 7, characterized in that the stabilizing agent is the disodium salt of glutaric, succinic or malonic acid whereby the concentration of the salt in the final formulation ranges between 10 and 30% (w/w).
9. Enzyme formulation according to any of claims 5 to 8, characterized in that the stabilizing agent is carboxymethyl-cellulose whereby the concentration of the polymer in the final formulation ranges between 1 and 10% (w/w).
10. Enzyme formulation according to any of claims 5 to 9, characterized in that the stabilizing agent is sodium alginate whereby the concentration of the polymer in the final formulation ranges between 1 and 10% (w/w).
11. Enzyme formulation according to any of claims 1-4, characterized in that the formulation is dry/solid.
12. Enzyme formulation according to claim 11, characterized in that the stabilizing agent is polyethylene glycol whereby the polyethylene glycol is present in a concentration of 1-20% (w/w) in the final formulation.
13. Enzyme formulation according to claim 11 or 12, characterized in that the stabilizing agent is xylitol and/or ribitol which is present in the final formulation in a concentration of 1-20% (w/w).
14. Enzyme formulation according to any of claims 11 to 13, characterized in that the stabilizing agent is the disodium salt of glutaric, succinic or malonic acid whereby the concentration of the salt in the final formulation ranges between 1 and 20% (w/w).
15. Enzyme formulation according to any of claims 11 to 14, characterized in that the stabilizing agent is carboxymethylcellulose whereby the concentration of the polymer in the final formulation ranges between 1 and 10% (w/w).
16. Enzyme formulation according to any of claims 11 to 15, characterized in that the stabilizing agent is sodium alginate whereby the concentration of the polymer in the final formulation ranges between 1 and 10% (w/w).
17. Enzyme formulation according to any of claims 2-5 or 11 characterized in that the phytase monomers are crosslinked by addition of glutaraldehyde.
18. Enzyme formulation according to any of claims 2-5 or 11 characterized in that the phytase monomers are crosslinked by oxidation of carbohydrate residues with sodium periodate and subsequent addition of adipic acid dihydrazide.
19. A method of preparing a feed composition for monogastric animals, characterized in that the feed is treated with a stabilized dry or liquid enzyme formulation according to any of claims 1-18.
20. A feed composition for monogastric animals, characterized in that the feed comprises a stabilized dry or liquid enzyme formulation according to any one of claims 1-18.
21. A method of providing a monogastric animal with its dietary requirement of phosphorous, characterized in that the animal is fed with a feed according to claim 20 and that no additional phosphorous is added to the feed.
22. A method of preparing a dry or liquid phytase formulation, characterized in that a stabilized phytase according to claims 1-18 is used.

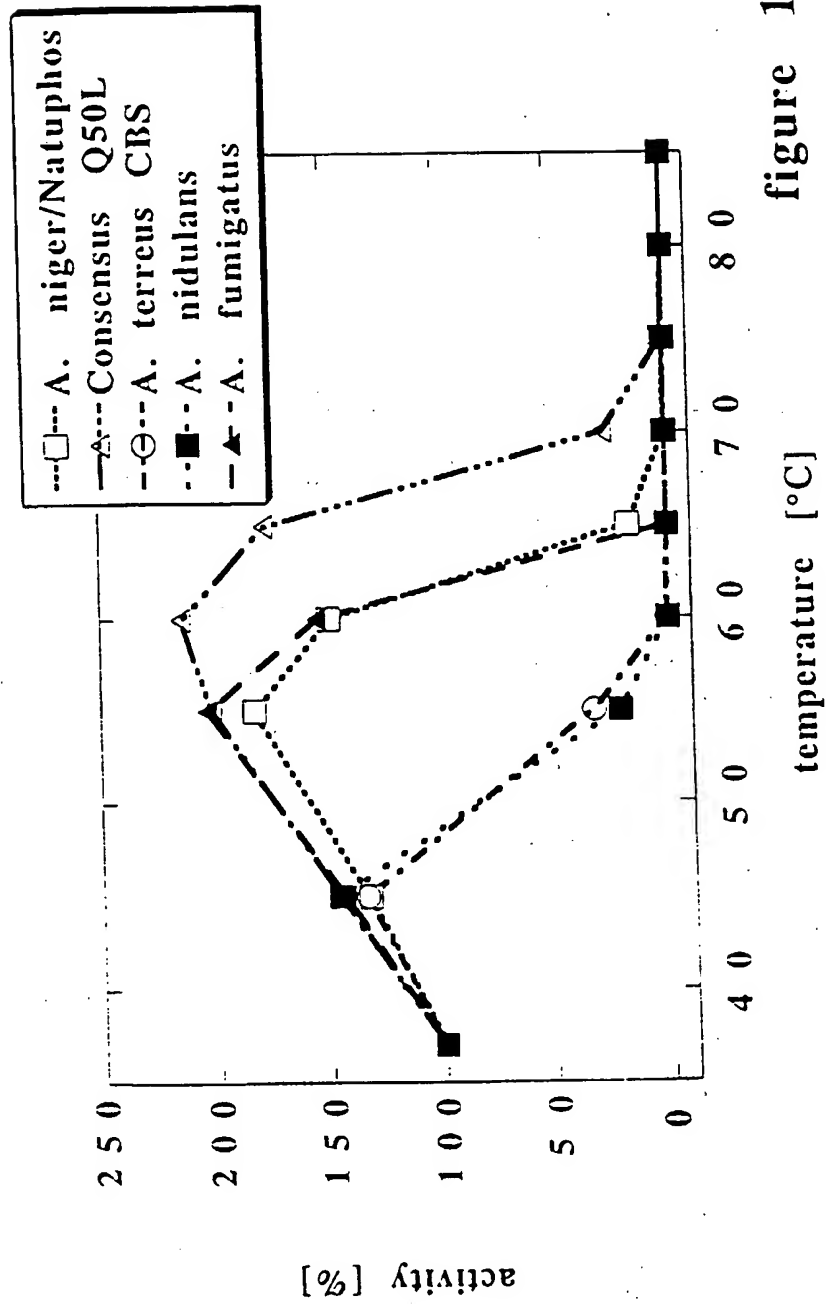
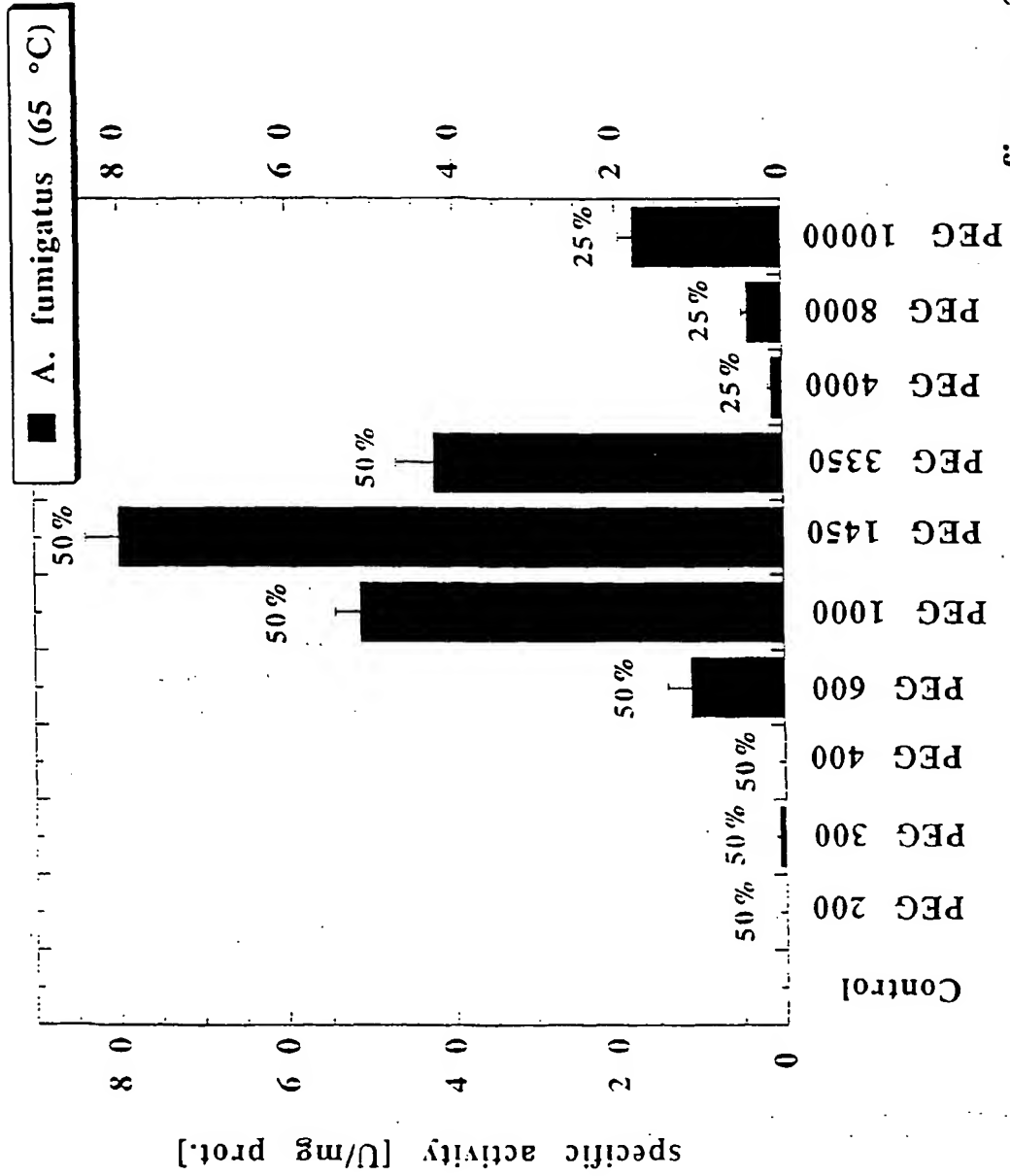


figure 1

figure 2



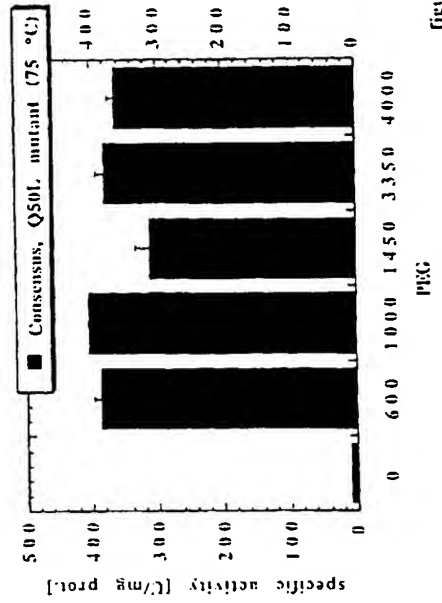


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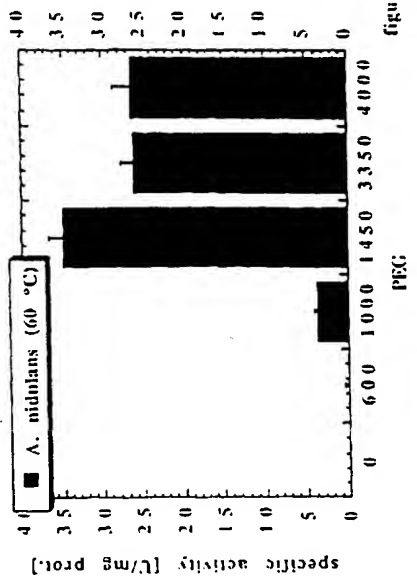


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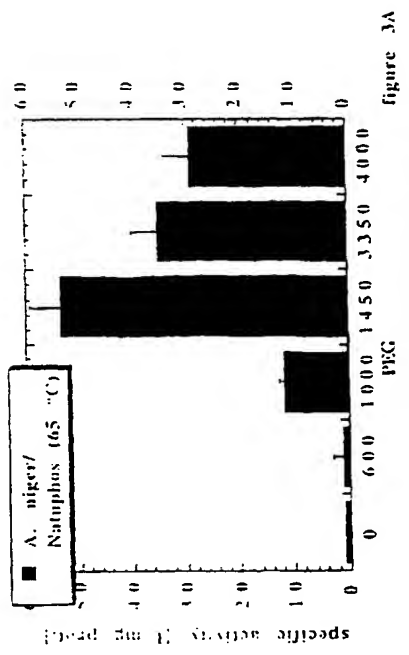


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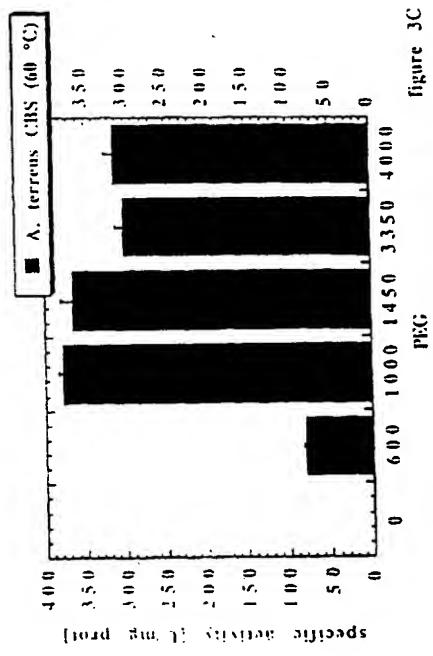
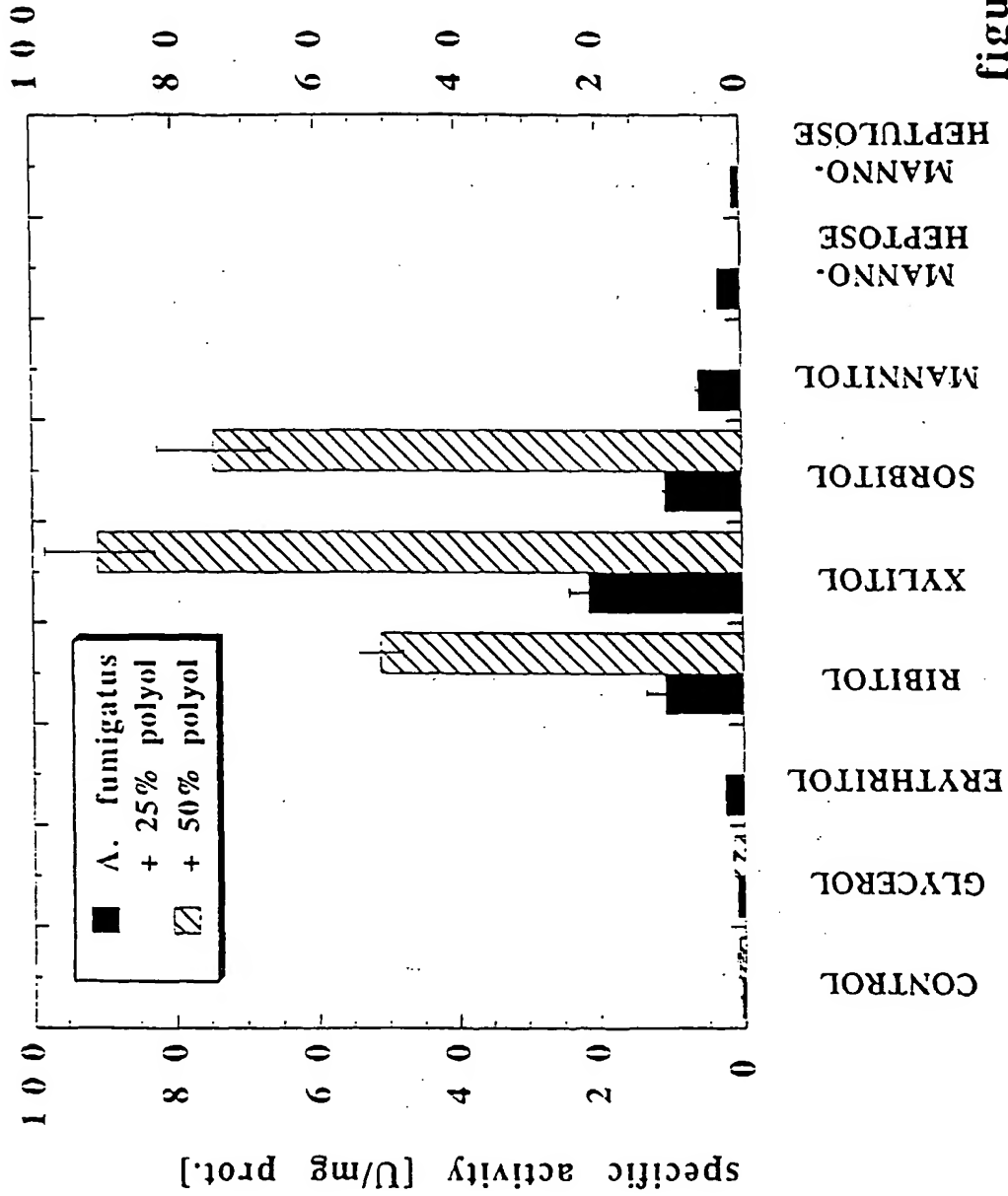


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figure 3

figure 4



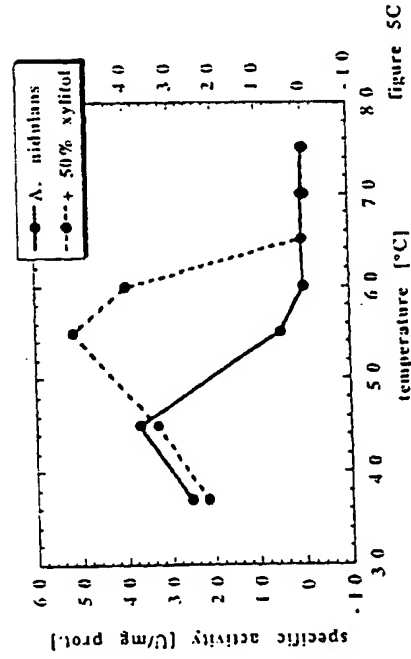
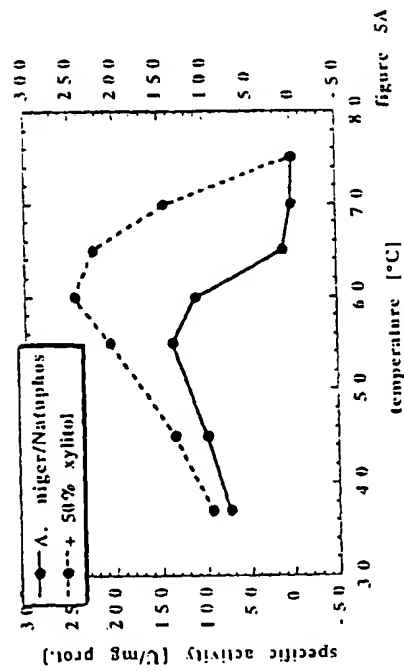
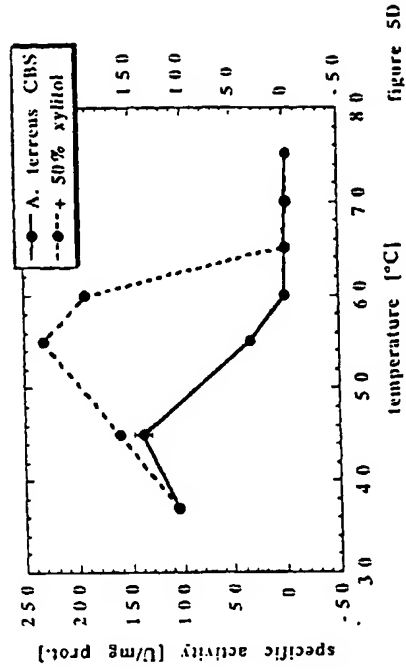
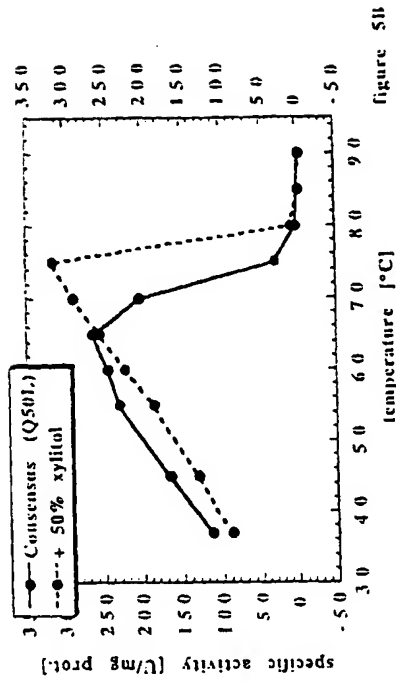


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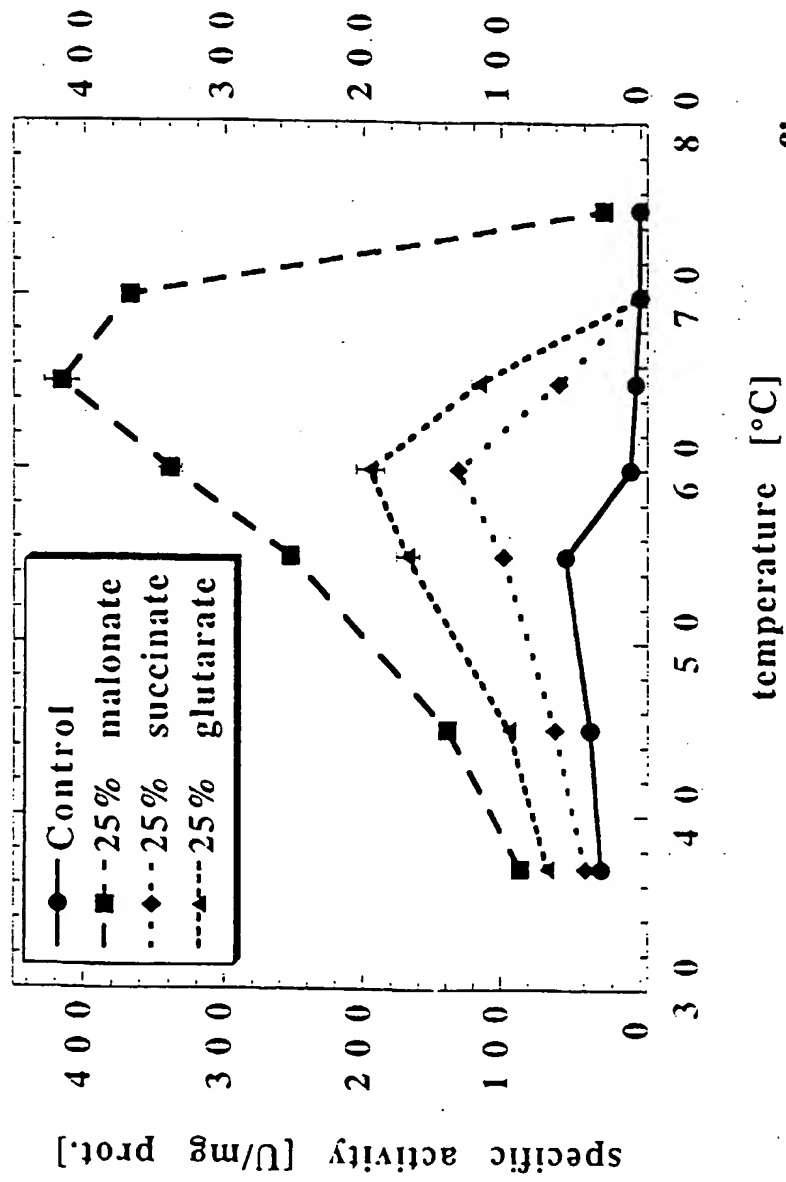


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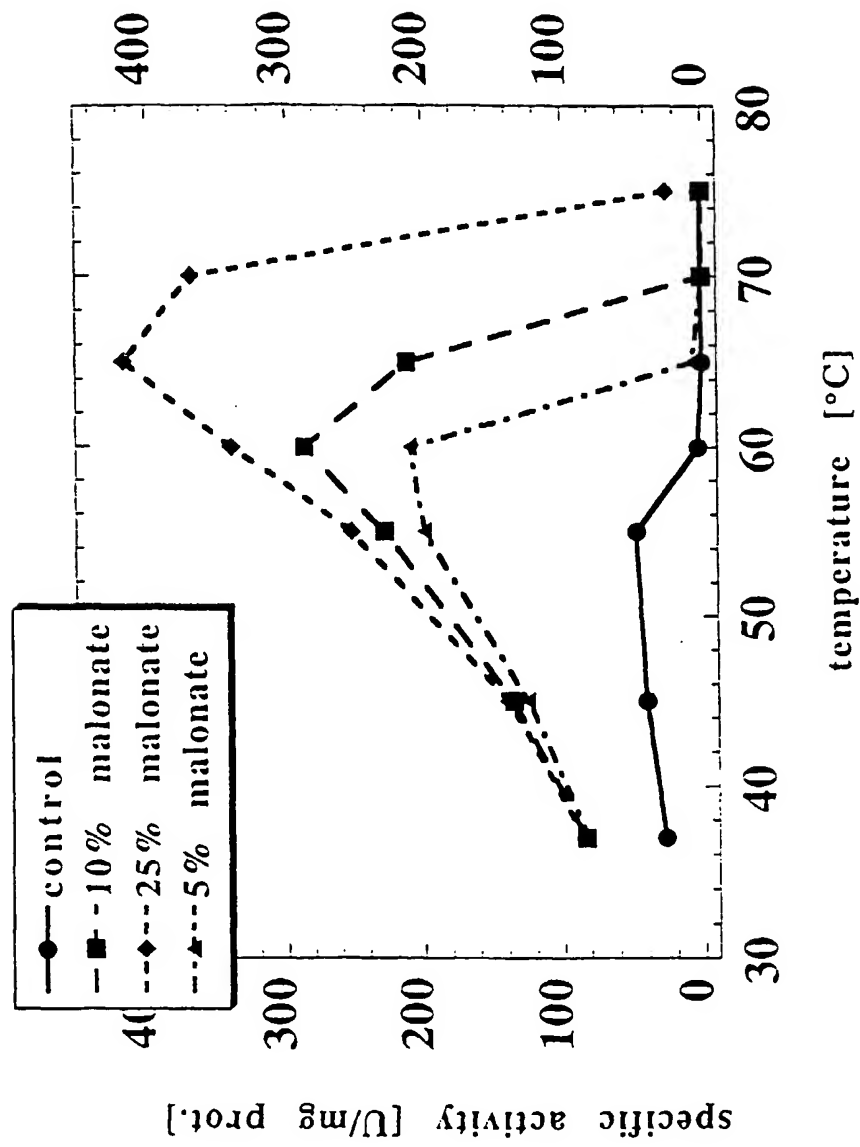


figure 7



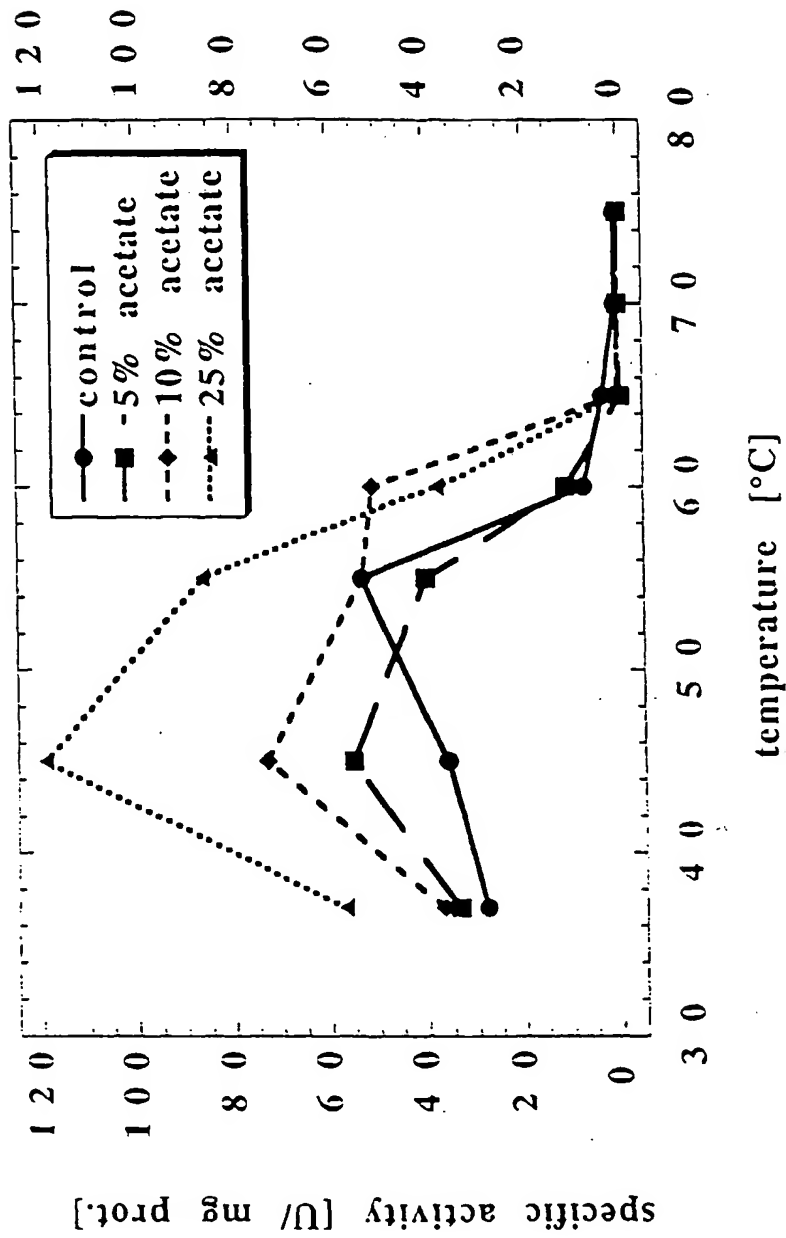


figure 8

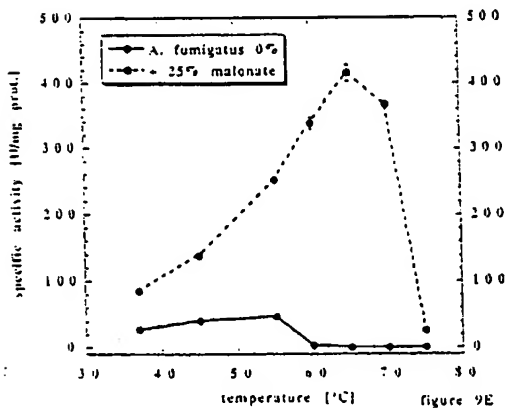
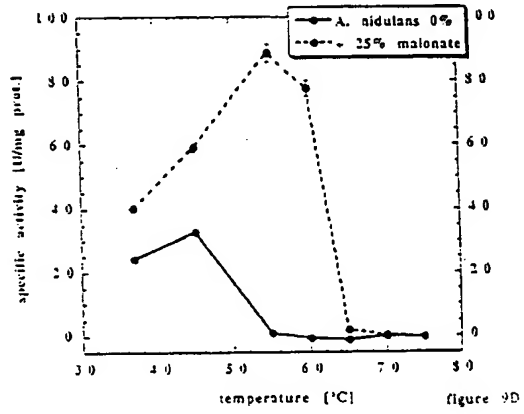
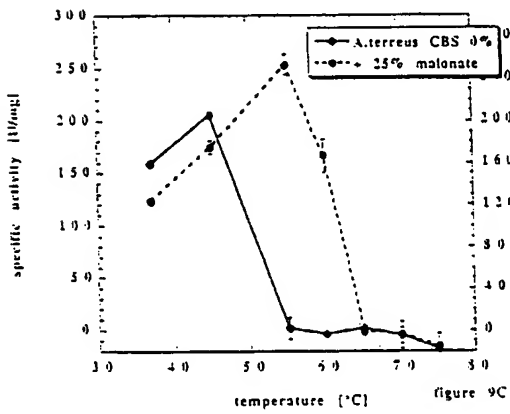
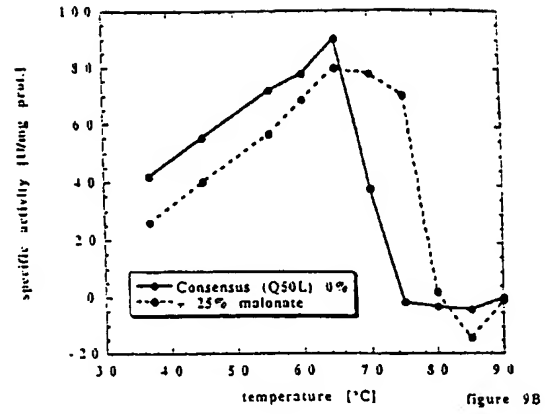
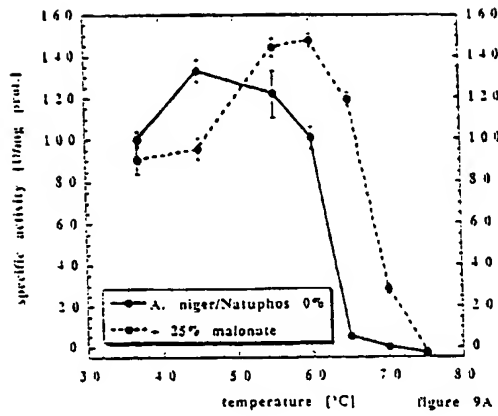


Figure 9

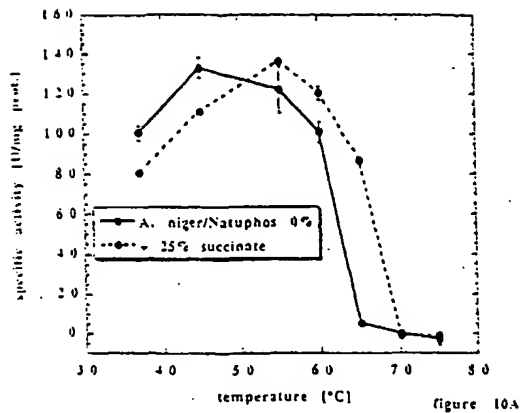


figure 10A

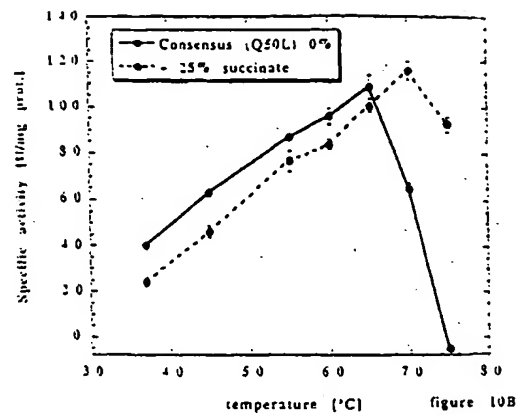


figure 10B

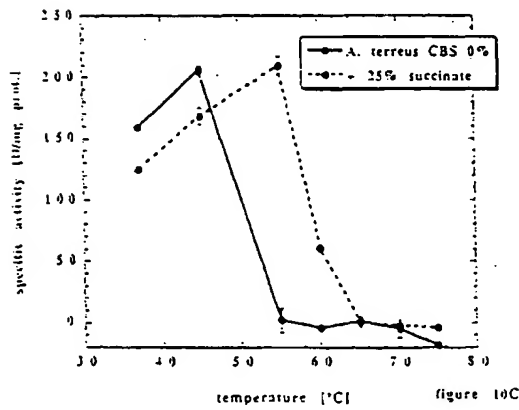


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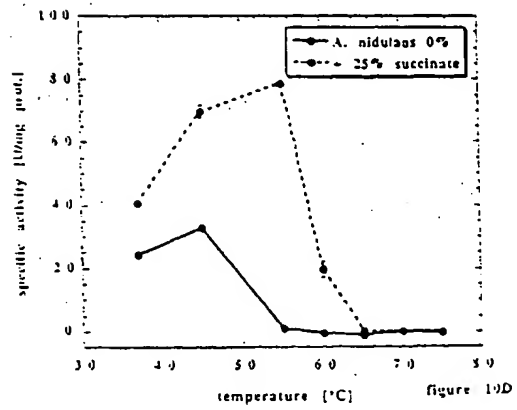


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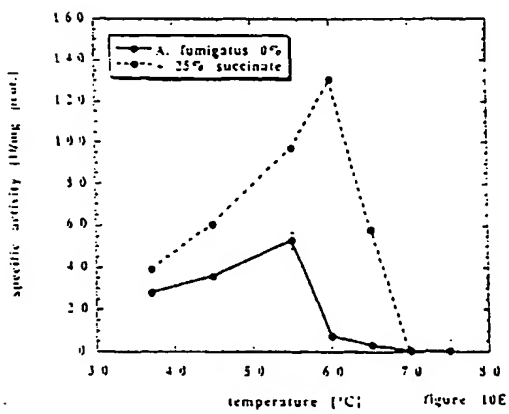


figure 10E

Figure 10

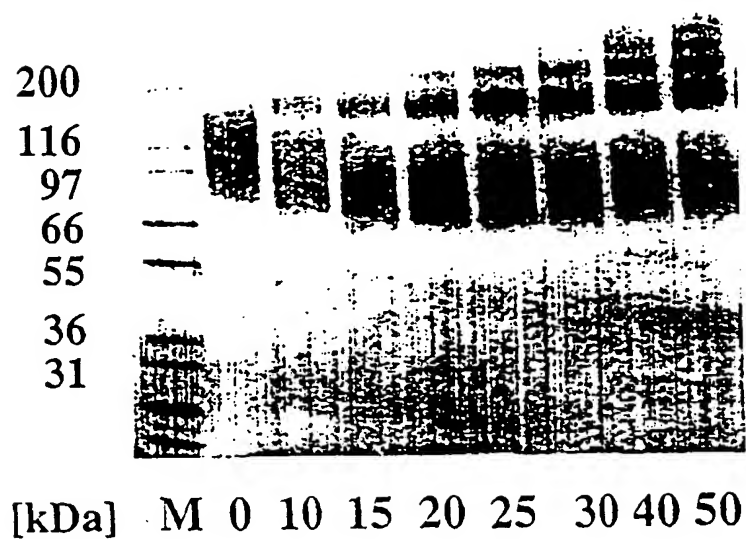


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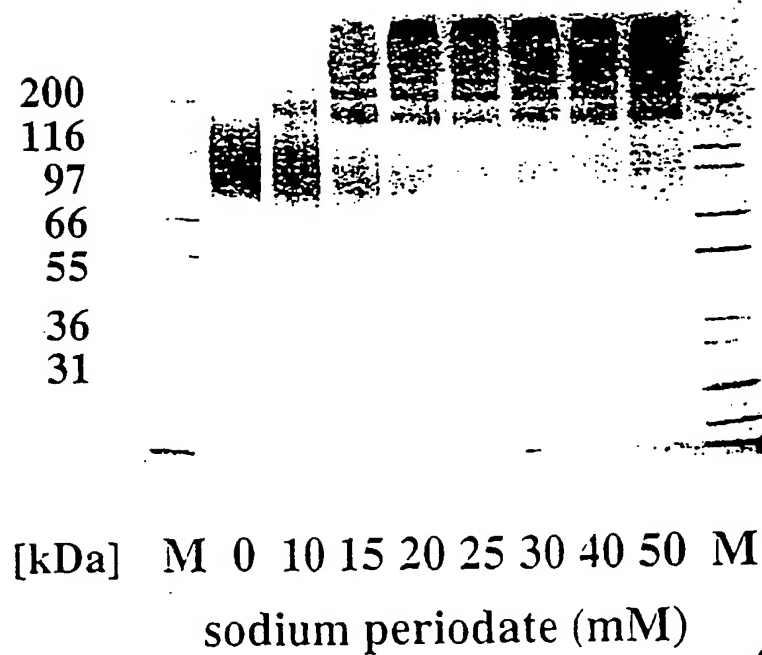


figure 11B

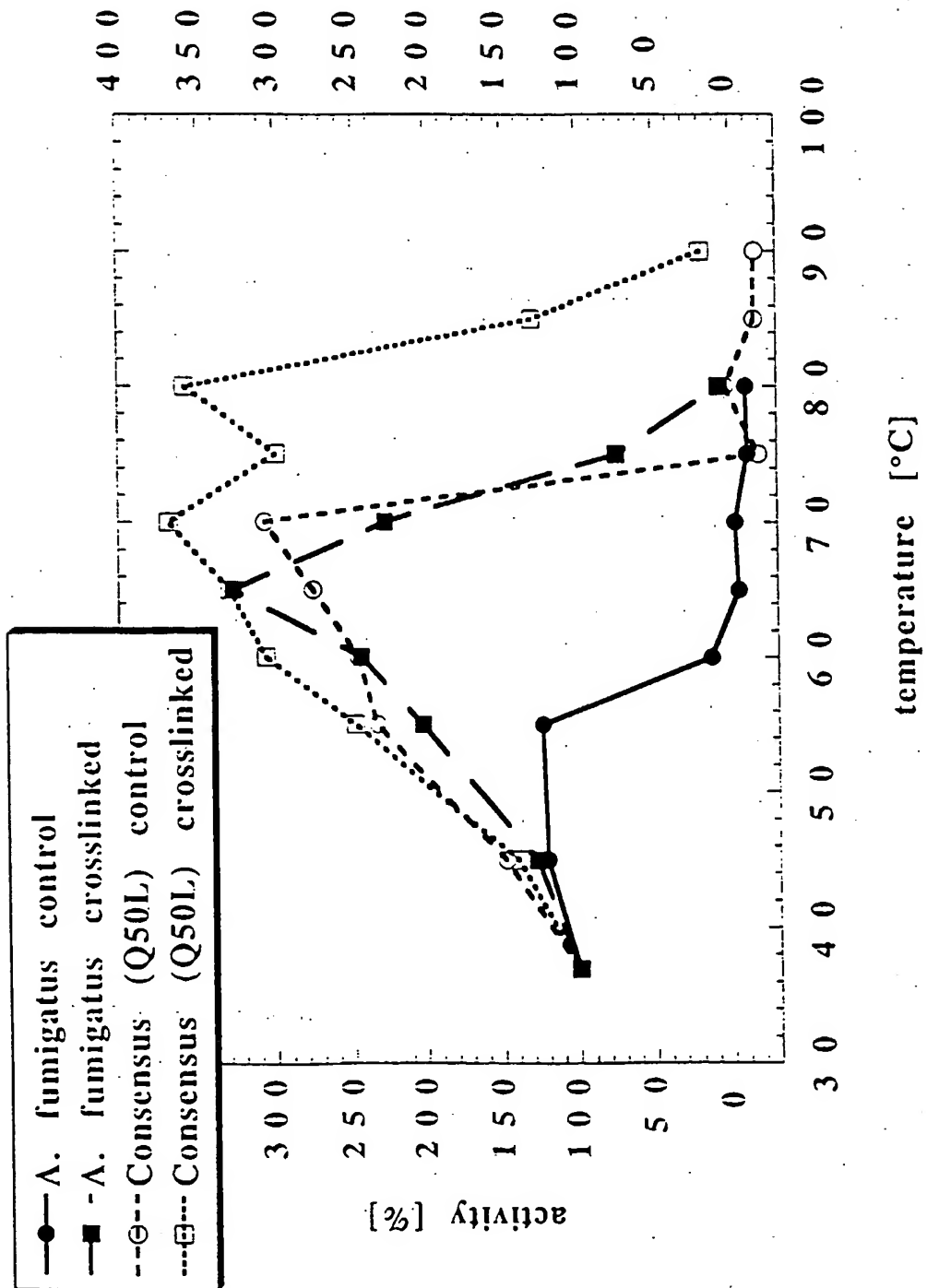


figure 12

Figure 13

	1
50	
<i>A. terreus</i> 9A-1	KhsDCNSVDh GYQCFPELSH kWGLYAPYFS
LQDESPFP1D VPEDChITFV	
<i>A. terreus</i> cbs	NhsDCTSVDr GYQCFPELSH kWGLYAPYFS
LQDESPFP1D VPDDChITFV	
<i>A. niger</i> var. <i>awamori</i>	NqstCDTVDQ GYQCFSETSH LWGQYAPFFS
LANESAISPD VPAGCrVTFA	
<i>A. niger</i> T213	NqsSCDTVDQ GYQCFSETSH LWGQYAPFFS
LANESVISPD VPAGCrVTFA	
<i>A. niger</i> NRRL3135	NqsSCDTVDQ GYQCFSETSH LWGQYAPFFS
LANESVISPE VPAGCrVTFA	
<i>A. fumigatus</i> 13073	GskSCDTVD1 GYQCsPATSH LWGQYSPFFS
LEDELSVSSK LPKDCrITLV	
<i>A. fumigatus</i> 32722	GskSCDTVD1 GYQCsPATSH LWGQYSPFFS
LEDELSVSSK LPKDCrITLV	
<i>A. fumigatus</i> 58128	GskSCDTVD1 GYQCsPATSH LWGQYSPFFS
LEDELSVSSK LPKDCrITLV	
<i>A. fumigatus</i> 26906	GskSCDTVD1 GYQCsPATSH LWGQYSPFFS
LEDELSVSSK LPKDCrITLV	
<i>A. fumigatus</i> 32239	GskACDTVE1 GYQCsPGTSH LWGQYSPFFS
LEDELSVSSD LPKDCrVTFV	
<i>E. nidulans</i>	QNHSCNTADG GYQCFPNVSH VWGQYSPYFS
IEQESAISd VPHGCEvTFV	
<i>T. thermophilus</i>	DSHSCNTVEG GYQCrPEISH sWGQYSPFFS
LADQSEISPD VPQNCkITFV	
<i>M. thermophila</i>	ESRPCDTpD1 GFQCgTAISH FWGQYSPYFS
VpSElDaS.. IPDDCEvTFA	
Consensus	NSHSCDTVDG GYQCFPEISH LWGQYSPYFS
LEDESAISPD VPDDC-VTFV	
Consensus phytase	NSHSCDTVDG GYQCFPEISH LWGQYSPYFS
LEDESAISPD VPDDCrVTFV	
	51
100	
<i>A. terreus</i> 9A-1	QVLARHGARS PThSKtKAYA AtIAAIQKSA
TaFpGKYAFL QSYNYSLDSE	
<i>A. terreus</i> cbs	QVLARHGARS PTDSKtKAYA AtIAAIQKNA
TaLpGKYAFL KSYNYSMGSE	
<i>A. niger</i> var. <i>awamori</i>	QVLSRHGARY PTESKgKkYS ALIEEIQQNV
TtFDGKYAFL KTYNYSLGAD	
<i>A. niger</i> T213	QVLSRHGARY PTESKgKkYS ALIEEIQQNV
TtFDGKYAFL KTYNYSLGAD	
<i>A. niger</i> NRRL3135	QVLSRHGARY PTDSKgKkYS ALIEEIQQNA
TtFDGKYAFL KTYNYSLGAD	
<i>A. fumigatus</i> 13073	QVLSRHGARY PTSSKsKkYK kLVTAIQaNA
TdFKGKFAFL KTYNYTLGAD	
<i>A. fumigatus</i> 32722	QVLSRHGARY PTSSKsKkYK kLVTAIQaNA
TdFKGKFAFL KTYNYTLGAD	
<i>A. fumigatus</i> 58128	QVLSRHGARY PTSSKsKkYK kLVTAIQaNA
TdFKGKFAFL KTYNYTLGAD	
<i>A. fumigatus</i> 26906	QVLSRHGARY PTSSKsKkYK kLVTAIQaNA
TdFKGKFAFL KTYNYTLGAD	
<i>A. fumigatus</i> 32239	QVLSRHGARY PTASKsKkYK kLVTAIQKNA
TeFKGKFAFL ETYNYTLGAD	
<i>E. nidulans</i>	QVLSRHGARY PTESKsKAYS GLIEAIQKNA
TsFwGQYAFL ESYNYTLGAD	

<i>T. thermophilus</i>	QLLSRHGARY PTSSKtElyS QLISrIQKTA
TaYKGyYAFL KDYrYqLGAN	
<i>M. thermophila</i>	QVLSRHGARa PTlKRaaSYv DLIDrIHhGA
IsYgPgYEFL RTYDYTLGAD	
Consensus	QVLSRHGARY PTSSK-KAYS ALIEAIQKNA T-
FKGKYAFL KTYNYTLGAD	
Consensus phytase	QVLSRHGARY PTSSKSKAYS ALIEAIQKNA
TAFKGKYAFL KTYNYTLGAD	

101

150

<i>A. terreus</i> 9A-1	ELTPFGrNQL rDlGaQFYeR YNALTRhInP
FVRATDASRV hESAeKFVEG	
<i>A. terreus</i> cbs	NLTPFGrNQL qDlGaQFYRR YDTLTRhInP
FVRAADSSRV hESAeKFVEG	
<i>A. niger</i> var. <i>awamori</i>	DLTPFGEQEL VNSGIKfYQR YESLTRNIIP
FIRSSGSSRV IASGEKFIEG	
<i>A. niger</i> T213	DLTPFGEQEL VNSGIKfYQR YESLTRNIIP
FIRSSGSSRV IASGEKFIEG	
<i>A. niger</i> NRRL3135	DLTPFGEQEL VNSGIKfYQR YESLTRNIVP
FIRSSGSSRV IASGKKFIEG	
<i>A. fumigatus</i> 13073	DLTPFGEQQL VNSGIKfYQR YKALARSVVP
FIRASGSDRV IASGEKFIEG	
<i>A. fumigatus</i> 32722	DLTPFGEQQL VNSGIKfYQR YKALARSVVP
FIRASGSDRV IASGEKFIEG	
<i>A. fumigatus</i> 58128	DLTPFGEQQL VNSGIKfYQR YKALARSVVP
FIRASGSDRV IASGEKFIEG	
<i>A. fumigatus</i> 26906	DLTAFGEQQL VNSGIKfYQR YKALARSVVP
FIRASGSDRV IASGEKFIEG	
<i>A. fumigatus</i> 32239	DLTPFGEQQM VNSGIKfYQK YKALAgSVVP
FIRSSGSDRV IASGEKFIEG	
<i>E. nidulans</i>	DLTiFGENQM VDSGaKFYRR YKNLARKnTP
FIRASGSDRV VASAeKFING	
<i>T. thermophilus</i>	DLTPFGENQM IQlGIKfYnH YKSLARNaVP
FVRCSGSDRV IASGrIFIEG	
<i>M. thermophila</i>	ELTRtGQQQM VNSGIKfYRR YRALARKsIP
FVRTAGqDRV VhSAENFTQG	
Consensus	DLTPFGENQM VNSGIKfYRR YKALARK-VP
FVRASGSDRV IASAeKFIEG	
Consensus phytase	DLTPFGENQM VNSGIKfYRR YKALARKIVP
FIRASGSDRV IASAeKFIEG	

151

200

*A. terreus* 9A-1 FQTARqDDHh ANpHQPSPrV DVaIPEGSAY  
 NNTLEHS1CT AFES...STV  
*A. terreus* cbs FQNARqGDPH ANpHQPSPrV DVVIPEGTAY  
 NNTLEHS1CT AFEA...STV  
*A. niger* var. *awamori* FQSTKLkDPr AqpgQSSPkI DVVISEASSs  
 NNTLDPGTCT VFED...SEL  
*A. niger* T213 FQSTKLkDPr AqpgQSSPkI DVVISEASSs  
 NNTLDPGTCT VFED...SEL  
*A. niger* NRRL3135 FQSTKLkDPr AqpgQSSPkI DVVISEASSs  
 NNTLDPGTCT VFED...SEL  
*A. fumigatus* 13073 FQqAKLADPG A.TNRAAPAI SVIIPESETF  
 NNTLDHGVCT kFEA...SQL  
*A. fumigatus* 32722 FQqAKLADPG A.TNRAAPAI SVIIPESETF  
 NNTLDHGVCT kFEA...SQL  
*A. fumigatus* 58128 FQqAKLADPG A.TNRAAPAI SVIIPESETF  
 NNTLDHGVCT kFEA...SQL  
*A. fumigatus* 26906 FQqAKLADPG A.TNRAAPAI SVIIPESETF  
 NNTLDHGVCT kFEA...SQL  
*A. fumigatus* 32239 FQqANVADPG A.TNRAAPVI SVIIPESETY  
 NNTLDHSVCT NFEA...SEL  
*E. nidulans* FRKAQLhDHG S..gQATPVV NVIIPEiDGF  
 NNTLDHSTCV SFEN...DER  
*T. thermophilus* FQSAKVlDPh SDkHDAPPTI NVIIeEGPSY  
 NNTLDtGSCP VFED...SSg  
*M. thermophila* FHSALLADRG STvRPTlPyd mVVIPETAGa  
 NNTLHNDlCT AFEEgpySTI  
  
 Consensus FQSAKLADPG S-PHQASPVI NVIIPEGSGY  
 NNTLDHGTCT AFED---SEL  
 Consensus phytase FQSAKLADPG SQPHQASPVI DVIIPEGSGY  
 NNTLDHGTCT AFED...SEL

201

250

*A. terreus* 9A-1 GDDAVANFTA VFAPAIaQRL EADLPGVqLS  
 TDDVVnLMAM CPFETVS1TD  
*A. terreus* cbs GDAAADNFTA VFAPAIakRL EADLPGVqLS  
 ADDVVnLMAM CPFETVS1TD  
*A. niger* var. *awamori* ADTVEANFTA TFAPSIRQRL ENDLSGVTLT  
 DTEVTyLMDM CSFDTIStST  
*A. niger* T213 ADTVEANFTA TFAPSIRQRL ENDLSGVTLT  
 DTEVTyLMDM CSFDTIStST  
*A. niger* NRRL3135 ADTVEANFTA TFVPSIRQRL ENDLSGVTLT  
 DTEVTyLMDM CSFDTIStST  
*A. fumigatus* 13073 GDEVAANFTA lFAPDIRARA EkHLPgVTLT  
 DEDVVSLMDM CSFDTVARTS  
*A. fumigatus* 32722 GDEVAANFTA lFAPDIRARA EkHLPgVTLT  
 DEDVVSLMDM CSFDTVARTS  
*A. fumigatus* 58128 GDEVAANFTA lFAPDIRARA EkHLPgVTLT  
 DEDVVSLMDM CSFDTVARTS  
*A. fumigatus* 26906 GDEVAANFTA lFAPDIRARA KkHLPgVTLT  
 DEDVVSLMDM CSFDTVARTS  
*A. fumigatus* 32239 GDEVEANFTA lFAPAIRARI EkHLPgVqLT  
 DDDVVSLMDM CSFDTVARTA  
*E. nidulans* ADEiEANFTA IMGPPIrkRL ENDLPGIKLT  
 NENViYlMDM CSFDTMARTA  
*T. thermophilus* GHDAQEKFAK qFAPAIleKI KDHLPGVDLA  
 VSDVpyLMDL CPFETLARNh



*M. thermophila* GDDAQDTYIS TFAGPITARV NANLPGANLT  
 DADTVaLMDL CPFETVAsSS  
  
 Consensus GDDAEANFTA TFAPAIRARL EADLPGVTLT DEDVV-  
 LMDM CPFETVARTS  
 Consensus phytase GDDVEANFTA LFAPAIRARL EADLPGVTLT  
 DEDVVYLMDM CPFETVARTS

251

300

*A. terreus* 9A-1 ..... DAhTLSPFC DLFTaEWtq  
 YNYLlSLDKY YGYGGGNPLG  
*A. terreus* cbs ..... DAhTLSPFC DLFTaEWtq  
 YNYLlSLDKY YGYGGGNPLG  
*A. niger* var. awamori ..... vDTKLSPFC DLFTHdEWih  
 YDYLQSLkKY YGHGAGNPLG  
*A. niger* T213 ..... vDTKLSPFC DLFTHdEWih  
 YDYLRSkKY YGHGAGNPLG  
*A. niger* NRRL3135 ..... vDTKLSPFC DLFTHdEWin  
 YDYLQSLkKY YGHGAGNPLG  
*A. fumigatus* 13073 ..... DASQLSPFC QLFTHnEWkk  
 YNYLQSLGKY YGYGAGNPLG  
*A. fumigatus* 32722 ..... DASQLSPFC QLFTHnEWkk  
 YNYLQSLGKY YGYGAGNPLG  
*A. fumigatus* 58128 ..... DASQLSPFC QLFTHnEWkk  
 YNYLQSLGKY YGYGAGNPLG  
*A. fumigatus* 26906 ..... DASQLSPFC QLFTHnEWkk  
 YNYLQSLGKY YGYGAGNPLG  
*A. fumigatus* 32239 ..... DASELSPFC AIFTHnEWkk  
 YDYLQSLGKY YGYGAGNPLG  
*E. nidulans* ..... HGTELSPEC AIFTEkEWlq  
 YDYLQSLSKY YGYGAGSPLG  
*T. thermophilus* ..... TDT.LSPFC ALSTQeEWqa  
 YDYYQSLGKY YGnGGGNPLG  
*M. thermophila* sdpatadagg gNGrpLSPFC rLFSEsEWra  
 YDYLQSVGKW YGYGPGNPLG  
  
 Consensus ----- DATELSPFC ALFTE-EW--  
 YDYLQSLGKY YGYGAGNPLG  
 Consensus phytase ..... DATELSPFC ALFTHdEWRO  
 YDYLQSLGKY YGYGAGNPLG

301

350

*A. terreus* 9A-1 PVQGVGWaNE LMARLTRAPV HDHTCVNNTL  
 DASPATFPLN ATLYADFSHD  
*A. terreus* cbs PVQGVGWaNE LIARLTRSPV HDHTCVNNTL  
 DANPATFPLN ATLYADFSHD  
*A. niger* var. *awamori* PTQGVGYaNE LIARLTHSPV HDDTSSNHTL  
 DSNPATFPLN STLYADFSHD  
*A. niger* T213 PTQGVGYaNE LIARLTHSPV HDDTSSNHTL  
 DSNPATFPLN STLYADFSHD  
*A. niger* NRRL3135 PTQGVGYaNE LIARLTHSPV HDDTSSNHTL  
 DSSPATFPLN STLYADFSHD  
*A. fumigatus* 13073 PAQGIGFtNE LIARLTRSPV QDHTSTNsTL  
 vSNPATFPLN ATMYVDFSHD  
*A. fumigatus* 32722 PAQGIGFtNE LIARLTRSPV QDHTSTNsTL  
 vSNPATFPLN ATMYVDFSHD  
*A. fumigatus* 58128 PAQGIGFtNE LIARLTRSPV QDHTSTNsTL  
 vSNPATFPLN ATMYVDFSHD  
*A. fumigatus* 26906 PAQGIGFtNE LIARLTRSPV QDHTSTNsTL  
 vSNPATFPLN ATMYVDFSHD  
*A. fumigatus* 32239 PAQGIGFtNE LIARLTNSPV QDHTSTNsTL  
 DSDPATFPLN ATYVDFSHD  
*E. nidulans* PAQGIGFtNE LIARLTQSPV QDNTSTNHTL  
 DSNPATFPLD rKLYADFSHD  
*T. thermophilus* PAQGVGFvNE LIARMTSPV QDYTTVNHTL  
 DSNPATFPLN ATLYADFSHD  
*M. thermophila* PTQGVGFvNE LLARLAGvPV RDgTSTNRTL  
 DGDPrTFPLG rPLYADFSHD  
  
 Consensus PAQGVGF-NE LIARLTHSPV QDHTSTNHTL  
 DSNPATFPLN ATLYADFSHD  
 Consensus phytase PAQGVGFANE LIARLTRSPV QDHTSTNHTL  
 DSNPATFPLN ATLYADFSHD

351

400

*A. terreus* 9A-1 SNLVSIFWAL GLYNGTAPLS qTSVESVSQT  
 DGYAAAWTVP FAARAYVEMM  
*A. terreus* cbs SNLVSIFWAL GLYNGTkPLS qTTVEDITrT  
 DGYAAAWTVP FAARAYIEMM  
*A. niger* var. *awamori* NGIISILFAL GLYNGTkPLS TTTVENITQT  
 DGFSSAWTVP FASrlyVEMM  
*A. niger* T213 NGIISILFAL GLYNGTkPLS TTTVENITQT  
 DGFSSAWTVP FASrlyVEMM  
*A. niger* NRRL3135 NGIISILFAL GLYNGTkPLS TTTVENITQT  
 DGFSSAWTVP FASrlyVEMM  
*A. fumigatus* 13073 NSMVSIFFAL GLYNGTEPLS rTSVESaKEl  
 DGYSASWVVP FGARAYFetM  
*A. fumigatus* 32722 NSMVSIFFAL GLYNGTGPLS rTSVESaKEl  
 DGYSASWVVP FGARAYFetM  
*A. fumigatus* 58128 NSMVSIFFAL GLYNGTEPLS rTSVESaKEl  
 DGYSASWVVP FGARAYFetM  
*A. fumigatus* 26906 NSMVSIFFAL GLYNGTEPLS rTSVESaKEl  
 DGYSASWVVP FGARAYFetM  
*A. fumigatus* 32239 NGMIPIFFAM GLYNGTEPLS qTSeESTKES  
 NGYSASWAVP FGARAYFetM  
*E. nidulans* NSMISIFFAM GLYNGTQPLS mDSVESIQEm  
 DGYAASWTVP FGARAYFELM  
*T. thermophilus* NTMTSIFaAL GLYNGTAKLS TTEIKSIEET  
 DGYSAAWTVP FGGRAYIEMM

*M. thermophila* NDMMGVLGAL GaYDGVPPLD KTArrDpEEI  
GGYAASWAVP FAARIYVEKM

Consensus NSMISIFFAL GLYNGTAPLS TTSVESIEET  
DGYAASWTVP FGARAYVEMM  
Consensus phytase NSMISIFFAL GLYNGTAPLS TTSVESIEET  
DGYSASWTVP FGARAYVEMM

401

450  
*A. terreus* 9A-1 QC..... RAEKE PLVRVLVNDR  
 VMPLHGCPD KLGRCKrDAF  
*A. terreus* cbs QC..... RAEKQ PLVRVLVNDR  
 VMPLHGCAVD NLGRCKrDDF  
*A. niger* var. *awamori* QC..... QAEQE PLVRVLVNDR  
 VVPLHGCPID aLGRCTrDSF  
*A. niger* T213 QC..... QAEQE PLVRVLVNDR  
 VVPLHGCPID aLGRCTrDSF  
*A. niger* NRRL3135 QC..... QAEQE PLVRVLVNDR  
 VVPLHGCPVD aLGRCTrDSF  
*A. fumigatus* 13073 QC..... KSEKE PLVRALINDR  
 VVPLHGCDVD KLGRCKLNDF  
*A. fumigatus* 32722 QC..... KSEKE PLVRALINDR  
 VVPLHGCDVD KLGRCKLNDF  
*A. fumigatus* 58128 QC..... KSEKE SLVRALINDR  
 VVPLHGCDVD KLGRCKLNDF  
*A. fumigatus* 26906 QC..... KSEKE PLVRALINDR  
 VVPLHGCDVD KLGRCKLNDF  
*A. fumigatus* 32239 QC..... KSEKE PLVRALINDR  
 VVPLHGCAVD KLGRCKLKDF  
*E. nidulans* QC..... E.KKE PLVRVLVNDR  
 VVPLHGCAVD KFGRCTLDDW  
*T. thermophilus* QC..... DDSDE PVVRVLVNDR  
 VVPLHGCEVD SLGRCKrDDF  
*M. thermophila* RCsggggggg ggegrQEKDE eMVRVLVNDR  
 VMTLkGCGAD ErGMCTLerF  
 Consensus QC----- QAEKE PLVRVLVNDR  
 VVPLHGCAVD KLGRCKLDDF  
 Consensus phytase QC..... QAEKE PLVRVLVNDR  
 VVPLHGCAVD KLGRCKRDDF

451

471

*A. terreus* 9A-1 VAGLSFAQAG GNWADCF---  
*A. terreus* cbs VEGLSFARAG  
 GNWAEFCF---  
*A. niger* var. *awamori* VrGLSFARSG GDWAECsA--  
*A. niger* T213 VrGLSFARSG GDWAECFA--  
*A. niger* NRRL3135 VrGLSFARSG  
 GDWAECFA--  
*A. fumigatus* 13073 VKGLSWARSG GNWGEFCFS--  
*A. fumigatus* 32722 VKGLSWARSG GNWGEFCFS--  
*A. fumigatus* 58128 VKGLSWARSG GNWGEFCFS--  
*A. fumigatus* 26906 VKGLSWARSG GNWGEFCFS--  
*A. fumigatus* 32239 VKGLSWARSG  
 GNSEQSFS--  
*E. nidulans* VEGLNfARSG GNWkTCFT1--  
*T. thermophilus* VrGLSFARqG GNWEGCYAas e  
*M. thermophila* IESMAFARGN GKWD1CFA--  
 Consensus VEGLSFARSG GNWAEFCFA--  
 Consensus phytase VEGLSFARSG GNWAEFCFA..

Figure 14

CP-1  
 Eco RI M G V F V V L L S I A T L F G S T  
 TATATGAATTCATGGGCGTGTTCGTCGCTACTGTCCATTGCCACCTTGTTCGGTTCCA  
 1 -----+-----+-----+-----+-----+ 60  
 ATATACTTAAGTACCCGCACAAGCAGCACGATGACAGGTAACGGTGGAACAAGCCAAGGT  
  
 S G T A L G P R G N S H S C D T V D G G  
 CATCCGGTACCGCCTTGGGTCTCGTGGTAATTCTCACTCTTGTGACACTGTTGACGGTG  
 61 -----+-----+-----+-----+-----+  
 120 GTAGGCCATGGCGGAACCCAGGAGCACCATTAAGAGTGAGAACACTGTGACAACCTGCCAC  
 CP-2  
 CP-3  
 Y Q C F P E I S H L W G Q Y S P Y F S L  
 GTTACCAATGTTTCCAGAAATTTCTCACTTGTGGGGTCAATACTCTCCATACTTCTCTT  
 121 -----+-----+-----+-----+-----+  
 180 CAATGGTTACAAAGGGTCTTTAAAGAGTGAACACCCAGTTATGAGAGGTATGAAGAGAA  
  
 E D E S A I S P D V P D D C R V T F V Q  
 TGGAAGACGAATCTGCTATTTCTCCAGACGTTCCAGACGACTGTAGAGTTACTTTCTGTT  
 181 -----+-----+-----+-----+-----+  
 240 ACCTTCTGCTTAGACGATAAAGAGGCTCTGCAAGGTCTGCTGACATCTCAATGAAAGCAAG  
 CP-4  
 CP-5  
 V L S R H G A R Y P T S S K S K A Y S A  
 AAGTTTTGTCTAGACACGGTGTCTAGATACCCAACTTCTTCTAAGTCTAAGGCTTACTCTG  
 241 -----+-----+-----+-----+-----+  
 300 TTCAAAACAGATCTGTGCCACGATCTATGGGTGAAGAAGATTGAGATTCCGAATGAGAC  
  
 L I E A I Q K N A T A F K G K Y A F L K  
 CTTTGATTGAAGCTATTCAAAGAAGCGCTACTGCTTTCAAGGGTAAGTACGCTTTCTTGA  
 301 -----+-----+-----+-----+-----+  
 360 GAAACTAATTCGATAAGTTTTCTTGCATGACGAAAGTTCCCATTCATGCGAAAGAACT  
 CP-6  
 CP-7  
 T Y N Y T L G A D D L T P F G E N Q M V  
 AGACTTACAACCTACACTTTGGGTGCTGACGACTTGACTCCATTGCGGTGAAAACCAAATGG  
 361 -----+-----+-----+-----+-----+  
 420 TCTGAATGTTGATGTGAAACCCACGACTGCTGAACTGAGGTAAGCCACTTTTGGTTTACC  
  
 N S G I K F Y R R Y K A L A R K I V P F  
 TTAACCTCTGGTATTAAGTTCTACAGAAGATACAAGGCTTTGGCTAGAAAGATTGTTCCAT  
 421 -----+-----+-----+-----+-----+  
 480 AATTGAGACCATAATTCAAGATGTCTTCTATGTTCCGAAACCGATCTTTCTAACAAGGTA  
 CP-8  
 CP-9  
 I R A S G S D R V I A S A E K F I E G F  
 TCATTAGAGCTTCTGGTTCGACAGAGTTATTGCTTCTGCTGAAAAGTTTCATTGAAGGTT  
 481 -----+-----+-----+-----+-----+  
 540 AGTAATCTCGAAGACCAAGACTGTCTCAATAACGAAGACGACTTTTCAAGTAACCTCCAA  
  
 Q S A K L A D P G S Q P H Q A S P V I D  
 TCCAATCTGCTAAGTTGGCTGACCCAGGTTCTCAACCACACCAAGCTTCTCCAGTTATTG

1140

GACTGAAGAGAGTGCTGTTGAGATACTAAAGATAAAAGAAGCGAAACCCAAACATGTTGC  
 CP-18  
 CP-19  
 T A P L S T T S V E S I E E T D G Y S A  
 GTACTGCTCCATTGTCTACTACTTCTGTTGAATCTATTGAAGAACTGACGGTTACTCTG  
 1141 -----+-----+-----+-----+-----+-----+-----+  
 1200 CATGACGAGGTAACAGATGATGAAGACAACCTTAGATAACTTCTTTGACTGCCAATGAGAC  
 S W T V P F G A R A Y V E M M Q C Q A E  
 CTTCTTGGACTGTTCCATTTCGGTGCTAGAGCTTACGTTGAAATGATGCAATGTCAAGCTG  
 1201 -----+-----+-----+-----+-----+-----+-----+  
 1260 GAAGAACCTGACAAGGTAAGCCACGATCTCGAATGCAACTTTACTACGTTACAGTTCGAC  
 CP-20  
 CP-21  
 K E P L V R V L V N D R V V P L H G C A  
 AAAAGGAACCATTGGTTAGAGTTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTG  
 1261 -----+-----+-----+-----+-----+-----+-----+  
 1320 TTTTCCTTGGTAACCAATCTCAAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACAC  
 V D K L G R C K R D D F V E G L S F  
 A R  
 CTGTTGACAAGTTGGGTAGATGTAAGAGAGACGACTTCGTTGAAGGTTTGTCTTTTCGCTA  
 1321 -----+-----+-----+-----+-----+-----+-----+  
 1380 GACAACTGTTCAACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGAT  
 CP-22  
 S G G N W A E C F A \* Eco RI  
 GATCTGGTGGTAACTGGGCTGAATGTTTCGCTTAAGAATTCATATA  
 1381 -----+-----+-----+-----+-----+-----+-----+ 1426  
 CTAGACCACCATGACCCGACTTACAAAGCGAATTCTTAAGTATAT

Figure 15

	1
50	
<i>P. involutus</i> (phyA1)	SvP.KnTAPt FPIPeseQrn WSPYSPYFPL AeYkAPPAGC
QInQVNIQR	
<i>P. involutus</i> (phyA2)	SvP.RniAPK FSIPeseQrn WSPYSPYFPL AeYkAPPAGC
EInQVNIQR	
<i>T. pubescens</i>	hiPlRdTSAC LdVTrDvQqs WSmYSPYFPA AtYvAPPASC
QInQVHIQR	
<i>A. pediades</i>	GgvvQaTfvQ pfFPpQiQds WAAyTPYYPV qaYtPPPkDC
KItQVNIQR	
<i>P. lycii</i>	StQfsfvAAQ LPIPaQntsn WGPYdPFFPV EpYaAPPEGC
tVtQVNLIQR	
Basidio	S-P-R-TAAQ LPIP-Q-Q-- WSPYSPYFPV A-Y-APPAGC QI-
QVNIIQR	
	51
100	
<i>P. involutus</i> (phyA1)	HGARFPTSGA TTRIKAGLTK LQGVqnfTDA KFNFIKSfky
dLGnsDLVVF	
<i>P. involutus</i> (phyA2)	HGARFPTSGA ATRIKAGLSK LQSVqnfTDP KFDfIKSfTY
dLGtsDLVVF	
<i>T. pubescens</i>	HGARFPTSGA AKRIQTAVAK LKAAsnyTDP lLAFVtNyTY
sLGqDsLVeL	
<i>A. pediades</i>	HGARFPTSGA GTRIQAaVKK LQSAktyTDP RLDFLtnyTY
tLGhDDLVPF	
<i>P. lycii</i>	HGARWPTSGA rSRqvAAVAK IQmArpfTDP KYEFLnDfvy
kFGvADLLPF	
Basidio	HGARFPTSGA ATRIQAaVAK LQSA---TDP KLDFL-N-TY -LG-
DDLVPF	
	101
150	
<i>P. involutus</i> (phyA1)	GAAQsfdAGQ EAFARYSkLV SkNNLPFIRA dGSDRVVDSA
TNWTAGFAsA	
<i>P. involutus</i> (phyA2)	GAAQsfdAGl EVFARYSkLV SsDNLPFIRS dGSDRVVDTA
TNWTAGFAsA	
<i>T. pubescens</i>	GATQsSEAGQ EAFTRYsLV SaDELpFVRA SGSDRVVATA
nNWTAGFAIA	
<i>A. pediades</i>	GAlQSSQAGE ETFqRYSfLV SkENLPFVRA SSSNRVVDSA
TNWTEGFSaA	
<i>P. lycii</i>	GAnQShQTGt DmYTRYStLf egGDVPFVRA AGdQRVVDSS
TNWTAGFGdA	
Basidio	GA-QSSQAGQ EAFTRYs-LV S-DNLpFVRA SGSDRVVDSA
TNWTAGFA-A	
	151
200	
<i>P. involutus</i> (phyA1)	ShNTvqPkLn LILPQtGNDT LEDNMCPaAG DSDPQvNaWL
AVafPSITAR	
<i>P. involutus</i> (phyA2)	SrNAiqPkLd LILPQtGNDT LEDNMCPaAG ESDPQvDaWL
AsafPSVTAQ	
<i>T. pubescens</i>	SsNSitPvLs VIISEaGNDT LDDNMCPaAG DSDPQvNgWL
AqFAPPMTAR	
<i>A. pediades</i>	ShHvlnPiLf VILSEslNDT LDDaMCPnAG sSDPQtGiWt
SIYGTPIAnR	

*P. lycii* SgETv1PtLq VVLqEeGNcT LcNNMCPnEv DGDest.tWL  
GVFAPnITAR

Basidio S-NT--P-L- VILSE-GNDT LDDNMCP-AG DSDPQ-N-WL  
AVFAPPITAR

201

250

*P. involutus* (phyA1) LNAAAPSVNL TDtDAfNLvs LCAf1TVSke kksdFctLFE  
giPGsFeAFa

*P. involutus* (phyA2) LNAAAPGANL TDaDAfNLvs LCPFMtVSke qksdFctLFE  
giPGsFeAFa

*T. pubescens* LNAGAPGANL TDtDTyNLlt LCPFETVate rrSeFCDIYE  
elQAE.dAFa

*A. pediades* LNqqAPGANI TAAdvsNLip LCAFETivke tpSpFCNLf.

.tPEEFaqFe

*P. lycii* LNAAAPSANL SDsDaltLmd MCPFDTLsG naSpFCDLf.

.tAEEYvSYe

Basidio LNAAAPGANL TD-DA-NL-- LCPFETVS-E --S-FCDLFE --PEEF-  
AF-

251

300

*P. involutus* (phyA1) YgGDLDKfYG TGYGQeLGPV QGVGYVNELI ARLTnsAVRD  
NTQTNRTLDA

*P. involutus* (phyA2) YaGDLDKfYG TGYGQALGPV QGVGYINELL ARLTnsAVnd  
NTQTNRTLDA

*T. pubescens* YnADLDKfYG TGYGQPLGPV QGVGYINELI ARLTaQnVsD

HTQTNsTLDS

*A. pediades* YfGDLDKfYG TGYGQPLGPV QGVGYINELL ARLTempVRD

NTQTNRTLDS

*P. lycii* YyyDLdkYyG TGpGNALGPV QGVGYVNEll ARLTgQAVRD

ETQTNRTLDS

Basidio Y-GDLDKfYG TGYGQPLGPV QGVGYINELL ARLT-QAVRD  
NTQTNRTLDS

301

350

*P. involutus* (phyA1) SPvTFPLNKT FYADFSHDNl MVAVFSAMGL FrQPAPLsTS  
vPNPwRTWrT

*P. involutus* (phyA2) APdTFPLNKT MYADFSHDNl MVAVFSAMGL FrQSAPLsTS  
tPDPNRTWLT

*T. pubescens* SPeTFPLNRT LYADFSHDNQ MVAIFSAMGL FNQSAPLDPT

tPDPaRTFLV

*A. pediades* SPLTFPLDRS IYADLSHDNQ MIAIFSAMGL FNQSSPLDPS

fPNPKRTWVT

*P. lycii* dPaTFPLNRT FYADFSHDNt MVPIFAALGL FNAtA.LDPl

kPDeNRlWVd

Basidio SP-TFPLNRT FYADFSHDNQ MVAIFSAMGL FNQSAPLDPS -  
PDPNRTWVT

351

400

*P. involutus* (phyA1) SsLVPFSGRM VVERLsC..f GT.....tkv  
RVLVQDqVQP



<i>P. involutus</i> (phyA2)	SsVVPFSARM aVERLsC..a GT.....	.....tkV
RVLVQDqVQP		
<i>T. pubescens</i>	kKIVPFSARM VVERLdC..g GA.....	.....qsV
RLLVNDAVQP		
<i>A. pediades</i>	SRLtPFSARM VtERLlCqrd GTgsgggsri mrngnvqtfV	
RILVNDALQP		
<i>P. lycii</i>	SKLVPFSGHM tVEKLaC... ..	.....sgkeaV
RVLVNDAVQP		

Basidio	SKLVPFSAARM VVERL-C--- GT-----	-----V
RVLVNDAVQP		

	401			441
<i>P. involutus</i> (phyA1)	LEFCGGDrNG lCTLakFVES	QtFARsDGaG	DFEKCFATSa	~
<i>P. involutus</i> (phyA2)	LEFCGGDqDG lCALDkFVES	QaYARsGGaG	DFEKCLATTv	-
<i>T. pubescens</i>	LAFCGADtsG vCTLDAFVES	QaYARNDGEG	DFEKCFAT--	~
<i>A. pediades</i>	LKFCGGDmDS lCTLEAFVES	QkYAREDGQG	DFEKCFD---	~
<i>P. lycii</i>	LEFCGG.vDG vCeLsAFVES	QtYARENGQG	DFAKCgfvPs	e
Basidio	LEFCGGD-DG -CTLDAFVES	Q-YAREDGQG	DFEKCFATP-	-

Figure 16

	1	
50		
<i>A. terreus</i> 9a1	KhsdCNSVDh	GYQCfPELSH kWGLYAPYFS LqDESFPPlD
VPeDCHITFV		
<i>A. terreus</i> cbs	NhsdCtSVDr	GYQCfPELSH kWGLYAPYFS LqDESFPPlD
VPdDCHITFV		
<i>A. niger</i> var. <i>awamori</i>	NqsTCDTVdQ	GYQCfSetSH LWGQYAPFFS LANESAISPD
VPaGCRVTFa		
<i>A. niger</i> NRRL3135	NqsSCDTVdQ	GYQCfSetSH LWGQYAPFFS LANESvISPE
VPaGCRVTFa		
<i>A. fumigatus</i> 13073	GskSCDTVd1	GYQCSPatSH LWGQYSPFFS LEDElSVSSK
LPkDCRITLV		
<i>A. fumigatus</i> 32722	GskSCDTVd1	GYQCSPatSH LWGQYSPFFS LEDElSVSSK
LPkDCRITLV		
<i>A. fumigatus</i> 58128	GskSCDTVd1	GYQCSPatSH LWGQYSPFFS LEDElSVSSK
LPkDCRITLV		
<i>A. fumigatus</i> 26906	GskSCDTVd1	GYQCSPatSH LWGQYSPFFS LEDElSVSSK
LPkDCRITLV		
<i>A. fumigatus</i> 32239	GskACDTVEl	GYQCSPGtSH LWGQYSPFFS LEDElSVSSD
LPkDCRVTFV		
<i>E. nidulans</i>	QNHSCNTaDG	GYQCfPNVSH VWGQYSPYFS IEQESAISeD
VPhGCeVTFV		
<i>T. thermophilus</i>	DSHSCNTVEG	GYQCrPEISH SWGQYSPFFS LADQSEISPD
VPqNCKITFV		
<i>T. lanuginosa</i>	-----nvDIAR	hWGQYSPFFS LAEvSEISPA
VPkGCRVeFV		
<i>M. thermophila</i>	ESRPCDTpDl	GFQCgTAISH FWGQYSPYFS VPsElDaS..
IPdDCeVTFa		
Basidio	xSxPxrxTAA	qLPipxQxqx xWSPYSPYFP VAXyxA....
pPaGCQIxqV		
	Consensus	NSHSCDTVdG GYQC-PEISH LWGQYSPFFS LADESAISPD VP-
GCRVTfV		
	Fcp10	NSHSCDTVdG GYQCfPEISH LWGQYSPFFS LADESAISPD
VPKGCRVTfV		
	51	
100		
<i>A. terreus</i> 9a1	QVLARHGARS	PThSKTKaYA AtIaAIQKSA TaFpGKYAFL
QSYNYSLDSE		
<i>A. terreus</i> cbs	QVLARHGARS	PTdSKTKaYA AtIaAIQKNA TaLpGKYAFL
KSYNYSMGSE		
<i>A. niger</i> var. <i>awamori</i>	QVLSRHGARY	PTesKGKKYS ALIeEIQQNv TtFDGKYAFL
KTYNYSLGAD		
<i>A. niger</i> NRRL3135	QVLSRHGARY	PTdSKGKKYS ALIeEIQQNA TtFDGKYAFL
KTYNYSLGAD		
<i>A. fumigatus</i> 13073	QVLSRHGARY	PTSSKSKKYk kLVtAIQaNA TdFKGKFAFL
KTYNYTLGAD		
<i>A. fumigatus</i> 32722	QVLSRHGARY	PTSSKSKKYk kLVtAIQaNA TdFKGKFAFL
KTYNYTLGAD		
<i>A. fumigatus</i> 58128	QVLSRHGARY	PTSSKSKKYk kLVtAIQaNA TdFKGKFAFL
KTYNYTLGAD		
<i>A. fumigatus</i> 26906	QVLSRHGARY	PTSSKSKKYk kLVtAIQaNA TdFKGKFAFL
KTYNYTLGAD		
<i>A. fumigatus</i> 32239	QVLSRHGARY	PTASKSKKYk kLVtAIQKNA TeFKGKFAFL
ETYNITLGAD		
<i>E. nidulans</i>	QVLSRHGARY	PTesKSKaYS GLIeAIQKNA TsFwGQYAFL
ESYNYTLGAD		

<i>T. thermophilus</i>	QLLSRHGARY PTSSKTELYS qLIsrIQKtA TaYKGyYAFL
KdYrYqLGAN	
<i>T. lanuginosa</i>	QVLSRHGARY PTAhKSEvYA ELLqrIQDtA TeFKGDFAFLL
RdYayhLGAD	
<i>M. thermophila</i>	QVLSRHGARA PTlkRAAsYv DLIdrIHhGA isYgPgYEFL
RTYDYTLGAD	
Basidio	NIIqRHGARF PTSGaAtRiq AaVakLQsax xxtDPKLDLFL
xnxtYxLGxD	

Consensus	QVLSRHGARY PTSSKSKKYS ALI-AIQKNA T-FKGKYAFL
KTNYNTLGAD	
Fcp10	QVLSRHGARY PTSSKSKKYS ALIEAIQKNA TAFKGKYAFL
KTNYNTLGAD	

101

150

<i>A. terreus</i> 9a1	ELTPFGGrNQL rDlGaQFYeR YNAL.TRhIn PFVRATDAsR
VhESAeKFVE	
<i>A. terreus</i> cbs	NLTPFGGrNQL qDlGaQFYRR YDTL.TRhIn PFVRAADSsR
VhESAeKFVE	
<i>A. niger</i> var. <i>awamori</i>	DLTPFGEQEL VNSGIKFYQR YESL.TRnII PFIRSSGSsR
VIASGEKFIE	
<i>A. niger</i> NRRL3135	DLTPFGEQEL VNSGIKFYQR YESL.TRnIV PFIRSSGSsR
VIASGKKFIE	
<i>A. fumigatus</i> 13073	DLTPFGEQQL VNSGIKFYQR YKAL.ARsVV PFIRASGSdR
VIASGEKFIE	
<i>A. fumigatus</i> 32722	DLTPFGEQQL VNSGIKFYQR YKAL.ARsVV PFIRASGSdR
VIASGEKFIE	
<i>A. fumigatus</i> 58128	DLTPFGEQQL VNSGIKFYQR YKAL.ARsVV PFIRASGSdR
VIASGEKFIE	
<i>A. fumigatus</i> 26906	DLTAFGEQQL VNSGIKFYQR YKAL.ARsVV PFIRASGSdR
VIASGEKFIE	
<i>A. fumigatus</i> 32239	DLTPFGEQQM VNSGIKFYQK YKAL.AgsVV PFIRSSGSdR
VIASGEKFIE	
<i>E. nidulans</i>	DLTiFGENQM VDsgAKFYRR YKnL.Arknt PFIRASGSdR
VVASAEKFIN	
<i>T. thermophilus</i>	DLTPFGENQM IQlGIKFYnH YKSL.ARnaV PFVRCsGSdR
VIASGrIFIE	
<i>T. lanuginosa</i>	NLTRFGEEQM MESGrQFYHR YREq.AReIV PFVRAAGSAR
VIASAEfFnr	
<i>M. thermophila</i>	ELTRtGQQQM VNSGIKFYRR YRAL.ARksI PFVRTAGqDR
VVhSAENftQ	
Basidio	DLvPFGAxQs sQAGqEaFtR YsxLvSxdnL PFVRASGSdR
VVDSAtNwtA	

Consensus	DLTPFGEQQM VNSGIKFYRR YKAL-AR-IV PFVRASGSdR
VIASAEKFIE	
Fcp10	DLTPFGEQQM VNSGIKFYRR YKAL.ARKIV PFVRASGSdR
VIASAEKFIE	

151

200

<i>A. terreus</i> 9a1	GFQTARqDDh hAnphQPSPr VDVaIPEGsA YNNTLEHSLC
TAFES...St	
<i>A. terreus</i> cbs	GFQNARqGDP hAnphQPSPr VDVVIPEGtA YNNTLEHSIC
TAFEA...St	

*A. niger* var. *awamori* GFQSTKLkDP rAqpgQSSPk IDVWISEAsS sNNTLDpGtC  
 TvFEa...SE  
*A. niger* NRRL3135 GFQSTKLkDP rAqpgQSSPk IDVWISEAsS sNNTLDpGtC  
 TvFEa...SE  
*A. fumigatus* 13073 GFQqAKLADP gAt.nRAAPa ISVIIPESeT FNNTLDHGVC  
 TkFEa...SQ  
*A. fumigatus* 32722 GFQqAKLADP gAt.nRAAPa ISVIIPESeT FNNTLDHGVC  
 TkFEa...SQ  
*A. fumigatus* 58128 GFQqAKLADP gAt.nRAAPa ISVIIPESeT FNNTLDHGVC  
 TkFEa...SQ  
*A. fumigatus* 26906 GFQqAKLADP gAt.nRAAPa ISVIIPESeT FNNTLDHGVC  
 TkFEa...SQ  
*A. fumigatus* 32239 GFQqANVADP gAt.nRAAPV ISVIIPESeT YNNTLDHSVC  
 TnFEa...SE  
*E. nidulans* GFRkaQLhDh g.s.gQATPV VNVIIEIdG FNNTLDHStC  
 vSFEn...dE  
*T. thermophilus* GFQSAKVlDP hSdKhDAPpT INVIIeEGpS YNNTLDtGsC  
 PvFEa...Ss  
*T. lanuginosa* GFQdAKdrDP rSnkdQAePV INVIISEeTg sNNTLDgltC  
 PAaEe...Ap  
*M. thermophila* GFHSALLADR gStvrPTlPy dmVVIPETaG aNNTLHNDLC  
 TAFEegPySt  
 Basidio GFaxA..... ..sxntxxPx LxVILSExg. .NDTLDDNMC  
 .....PxAG  
  
 Consensus GFQSAKLADP -A---QASPV INVIIPEG-G YNNTLDHGGLC  
 TAFE--P-SE  
 Fcp10 GFQSAKLADP GANPHQASPV INVIIPEGAG YNNTLDHGGLC  
 TAFEe...SE  
  
 201  
 250  
*A. terreus* 9a1 VGDDaVANFT AVFAPAIaQR LEAdLPGVQL StDDVVNLMA  
 MCPFETVSLT  
*A. terreus* cbs VGDAaADNFT AVFAPAIaKR LEAdLPGVQL SADDVVNLMA  
 MCPFETVSLT  
*A. niger* var. *awamori* LADtVEANFT AtFAPSIRqR LEndLSGVtL TDtEVtyLMD  
 MCSFDTISTs  
*A. niger* NRRL3135 LADtVEANFT AtFvPSIRqR LEndLSGVtL TDtEVtyLMD  
 MCSFDTISTs  
*A. fumigatus* 13073 LGDEVAANFT ALFAPdIRAR aEkhlPGVtL TDEDVVSLMD  
 MCSFDTVArT  
*A. fumigatus* 32722 LGDEVAANFT ALFAPdIRAR aEkhlPGVtL TDEDVVSLMD  
 MCSFDTVArT  
*A. fumigatus* 58128 LGDEVAANFT ALFAPdIRAR aEkhlPGVtL TDEDVVSLMD  
 MCSFDTVArT  
*A. fumigatus* 26906 LGDEVAANFT ALFAPdIRAR aKkhLPGVtL TDEDVVSLMD  
 MCSFDTVArT  
*A. fumigatus* 32239 LGDEVEANFT ALFAPAIRAR IEkhLPGVQL TDDDVVSLMD  
 MCSFDTVArT  
*E. nidulans* rADEIEANFT AIMGPPIRkR LEndLPGIKL TNENViyLMD  
 MCSFDTMArT  
*T. thermophilus* gGHDAQEKFA kqFAPAIEk IKDhLPGVDL AvsDVpyLMD  
 LCPFETLArN  
*T. lanuginosa* .DptqpAEf1 qVFGPRVlkK ItkhMPGVNL TLEDVp1FMD  
 LCPFDTVGsd  
*M. thermophila* IGDDaQDtYl StFAGPitAR VNAnLPGaNL TDADtVaLMD  
 LCPFETVAsS  
 Basidio dSDpqxnxWl AVFAPPitAR LNAaaPGaNL TDxDaxNLxx  
 LCPFETVS...

		Consensus	LGDDVEANFT AVFAPPPIRAR LEA-LPGVNL TDEDVVNLMD
MCPFDTV-A-T		Fcp10	LGDDVEANFT AVFAPPPIRAR LEAHLPGVNL TDEDVVNLMD
MCPFDTVART			
		251	
300			
A. terreus 9a1	dD..Aht...	LSPF	CDLFta..tE WtQYNYLlSL
dKYYGYGGGN			
A. terreus cbs	dD..Aht...	LSPF	CDLFta..aE WtQYNYLlSL
dKYYGYGGGN			
A. niger var. awamori	Tv..DTK...	LSPF	CDLFTH..dE WlHYDYLQSL
kKYYGHGAGN			
A. niger NRRL3135	Tv..DTK...	LSPF	CDLFTH..dE WlNYDYLQSL
kKYYGHGAGN			
A. fumigatus 13073	SD..ASQ...	LSPF	CQLFTH..nE WkKYNyLQSL
gKYYGYGAGN			
A. fumigatus 32722	SD..ASQ...	LSPF	CQLFTH..nE WkKYNyLQSL
gKYYGYGAGN			
A. fumigatus 58128	SD..ASQ...	LSPF	CQLFTH..nE WkKYNyLQSL
gKYYGYGAGN			
A. fumigatus 26906	SD..ASQ...	LSPF	CQLFTH..nE WkKYNyLQSL
gKYYGYGAGN			
A. fumigatus 32239	AD..ASE...	LSPF	CAIFTH..nE WkKYDYLQSL
gKYYGYGAGN			
E. nidulans	AH..GTE...	LSPF	CAIFTE..kE WlQYDYLQSL
sKYYGYGAGS			
T. thermophilus	ht..DT....	LSPF	CALStQ..eE WqayDYyQSL
gKYYGnGGGN			
T. lanuginosa	PvlfPrQ...	LSPF	CHLFta..dD WmaYDYyTL
dKYYSHGGGS			
M. thermophila	SsdpaTadag	ggngprLSPF	CrLFSE..sE WraYDYLQSV
gKWYGYGPGN			
Basidio	.....	...xexxSxF	CDLFexxpeE FxaFxyxgdL
dKFYGTyGyGQ			
		Consensus	SD--ATQ--- -----LSPF CDLFTH---E W-QYDYLQSL -
KYYGYGAGN			
		Fcp10	SD..ATQ... .....LSPF CDLFTH..DE WlQYDYLQSL
GKYYGYGAGN			
		301	
350			
A. terreus 9a1	PLGPvQGVGW	aNELMARLTR	A.PVHDHTCv NNTLDASPAT
FPLNATLYAD			
A. terreus cbs	PLGPvQGVGW	aNELIARLTR	S.PVHDHTCv NNTLDANPAT
FPLNATLYAD			
A. niger var. awamori	PLGPTQGVGY	aNELIARLTH	S.PVHDDTSS NHTLDSNPAT
FPLNSTLYAD			
A. niger NRRL3135	PLGPTQGVGY	aNELIARLTH	S.PVHDDTSS NHTLDSSPAT
FPLNSTLYAD			
A. fumigatus 13073	PLGPAQGIGF	tNELIARLTR	S.PVQDHTST NstLvSNPAT
FPLNATMYvD			
A. fumigatus 32722	PLGPAQGIGF	tNELIARLTR	S.PVQDHTST NstLvSNPAT
FPLNATMYvD			
A. fumigatus 58128	PLGPAQGIGF	tNELIARLTR	S.PVQDHTST NstLvSNPAT
FPLNATMYvD			
A. fumigatus 26906	PLGPAQGIGF	tNELIARLTR	S.PVQDHTST NstLvSNPAT
FPLNATMYvD			
A. fumigatus 32239	PLGPAQGIGF	tNELIARLTN	S.PVQDHTST NstLSDSPAT
FPLNATIYvD			

<i>E. nidulans</i>	PLGPAQGIGF tNELIARLTQ S.PVQDNTST NHTLDSNPAT
FPLDrkLYAD	
<i>T. thermophilus</i>	PLGPAQGVGF vNELIARMTH S.PVQDYTTv NHTLDSNPAT
FPLNATLYAD	
<i>T. lanuginosa</i>	AFGPSRGVGF vNELIARMTg NlPVKDHTTv NHTLDdNPET
FPLDAVLYAD	
<i>M. thermophila</i>	PLGPTQGVGF vNELLARLA. GvPVRDgTST NRTLGDGPtT
FPLGrPLYAD	
Basidio	PLGPvQGVGY iNELLARLTx qa.VRDNTqT NRTLDSsPxT
FPLNrTFYAD	
Consensus	PLGPAQGVGF -NELIARLTH S-PVQDHTST NHTLDSNPAT
FPLNATLYAD	
Fcp10	PLGPAQGVGF vNELIARLTH S.PVQDHTST NHTLDSNPAT
FPLNATLYAD	
	351
400	
<i>A. terreus</i> 9a1	FSHDSnLVSI FWALGLYNGT aPLSqtSVE. .SvsQTDGYA
AAWTVPFAAR	
<i>A. terreus</i> cbs	FSHDSnLVSI FWALGLYNGT kPLSqtTVE. .ditrTDGYA
AAWTVPFAAR	
<i>A. niger</i> var. <i>awamori</i>	FSHDNGIISI LFALGLYNGT kPLSTTTVE. .NitQTDGFS
SAWTVPFASR	
<i>A. niger</i> NRRL3135	FSHDNGIISI LFALGLYNGT kPLSTTTVE. .NitQTDGFS
SAWTVPFASR	
<i>A. fumigatus</i> 13073	FSHDNSMVISI FFALGLYNGT ePLSrTSVE. .SaKElDGYS
ASWvVPFGAR	
<i>A. fumigatus</i> 32722	FSHDNSMVISI FFALGLYNGT gPLSrTSVE. .SaKElDGYS
ASWvVPFGAR	
<i>A. fumigatus</i> 58128	FSHDNSMVISI FFALGLYNGT ePLSrTSVE. .SaKElDGYS
ASWvVPFGAR	
<i>A. fumigatus</i> 26906	FSHDNSMVISI FFALGLYNGT ePLSrTSVE. .SaKElDGYS
ASWvVPFGAR	
<i>A. fumigatus</i> 32239	FSHDNGMIPI FFAMGLYNGT ePLSqtSeE. .StKESNGYS
ASWAVPFGAR	
<i>E. nidulans</i>	FSHDNSMISI FFAMGLYNGT qPLSmdSVE. .SiQEmDGYA
ASWTVPFGAR	
<i>T. thermophilus</i>	FSHDNTMtSI FaALGLYNGT akLSTTeIK. .SiEETDGYS
AAWTVPFGGR	
<i>T. lanuginosa</i>	FSHDNTMtGI FsAMGLYNGT kPLSTSkIQP pTgAAADGYA
ASWTVPFAAR	
<i>M. thermophila</i>	FSHDNdMMGV LgALGaYDgV pPLdkTA..R rdpEElGGYA
ASWAVPFAAR	
Basidio	FSHDNqMVAI FsAMGLFNqS aPLdPSxpDP nrt....Wv
TSklVPFSAR	
Consensus	FSHDNTMVISI FFALGLYNGT -PLSTTSVEP -S-EETDGYA
ASWTVPFAAR	
Fcp10	FSHDNTMVISI FFALGLYNGT KPLSTTSVE. .SiEETDGYA
ASWTVPFAAR	
	401
450	
<i>A. terreus</i> 9a1	AYVEMMQC.. ra.....EKEPL VRVLVNDVRM
PLHGCPtDKL	
<i>A. terreus</i> cbs	AYIEMMQC.. ra.....EKQPL VRVLVNDVRM
PLHGCAVDNL	
<i>A. niger</i> var. <i>awamori</i>	lyVEMMQC.. Qa.....EQEPL VRVLVNDRVV
PLHGCPIDaL	
<i>A. niger</i> NRRL3135	lyVEMMQC.. Qa.....EQEPL VRVLVNDRVV
PLHGCPVDaL	

A. fumigatus 13073	AYfEtMQC.. Ks.....	.....EKEPL VRaLINDRVV
PLHGCDVDKL		
A. fumigatus 32722	AYfEtMQC.. Ks.....	.....EKEPL VRaLINDRVV
PLHGCDVDKL		
A. fumigatus 58128	AYfEtMQC.. Ks.....	.....EKESL VRaLINDRVV
PLHGCDVDKL		
A. fumigatus 26906	AYfEtMQC.. Ks.....	.....EKEPL VRaLINDRVV
PLHGCDVDKL		
A. fumigatus 32239	AYfEtMQC.. Ks.....	.....EKEPL VRaLINDRVV
PLHGCAVDKL		
E. nidulans	AYfELMQC.. E.....	.....KKEPL VRVLVNDRVV
PLHGCAVDKF		
T. thermophilus	AYIEMMQC.. Dd.....	.....sDEPV VRVLVNDRVV
PLHGCEVDsL		
T. lanuginosa	AYVELLRC.. Etetsseeee	EG...EDEPF VRVLVNDRVV
PLHGCrVDRW		
M. thermophila	iYVEkMRC.. sggggggggg	EGrqeKDEeM VRVLVNDRVM
TLkGCGaDEr		
Basidio	mvVErLxCxx	xgtxxxxxxxx xxxxxxxxxxx VRVLVNDaVq
PLEfCGgDxd		
Consensus	AYVEMMQC-- E-----	EG---EKEPL VRVLVNDRVV
PLHGCGVDKL		
Fcp10	AYVEMMQC.. EA.....	.....EKEPL VRVLVNDRVV
PLHGCGVDKL		

	451	482
<i>A. terreus</i> 9a1	GRCKrDAFVA GLSFAQAG..	GNWADCF--- --
<i>A. terreus</i> cbs	GRCKrDDFVE GLSFARAG..	GNWAECEf--- --
<i>A. niger</i> var. <i>awamori</i>	GRCtrDsFVr GLSFARSG..	GDWAECSA--- --
<i>A. niger</i> NRRL3135	GRCtrDsFVr GLSFARSG..	GDWAECEFA--- --
<i>A. fumigatus</i> 13073	GRCKlNDFVK GLSWARSG..	GNWGECEFS--- --
<i>A. fumigatus</i> 32722	GRCKlNDFVK GLSWARSG..	GNWGECEFS--- --
<i>A. fumigatus</i> 58128	GRCKlNDFVK GLSWARSG..	GNWGECEFS--- --
<i>A. fumigatus</i> 26906	GRCKlNDFVK GLSWARSG..	GNWGECEFS--- --
<i>A. fumigatus</i> 32239	GRCKlKDFVK GLSWARSG..	GNSEQSFS--- --
<i>E. nidulans</i>	GRCtlDDWVE GLNFARSG..	GNWktCFTl--- --
<i>T. thermophilus</i>	GRCKrDDFVr GLSFARqG..	GNWEGCYAas e-
<i>T. lanuginosa</i>	GRCRrDEWIK GLTFARqG..	GHWDrCF--- --
<i>M. thermophila</i>	GmCtlErFIE SMAFARGN..	GKWDlCFA--- --
Basidio	GxCtlDAFVE SqxYAReDgq	GDFEKCFatp xx
Consensus	GRCK-DDFVE GLSFARSG--	GNWEECEFA-- --
Fcp10	GRCKRDDFVE GLSFARSG..	GNWEECEFA.. ..



Figure 17

CP-1  
Eco RI M G V F V V L L S I A T L F G S T  
17  
TATATGAATTCATGGCGCTGTTTCGTGCTGCTACTGTCCATTGCCACCTTGTTCGGTTCCA  
1 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 60  
ATATACTTAAGTACCCGACACAAGCAGCAGCATGACAGGTAACGGTGGAAACAAGCCAAGGT  
  
S G T A L G P R G N S H S C D T V D G G  
37  
CATCCGGTACCGCCTTGGGTCTCGTGGTAATTCTCACTCTTGTGACACTGTTGACGGTG  
61 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+  
120  
GTAGGCCATGGCGGAACCCAGGAGCACCATTAAAGAGTGAGAACACTGTGACAACTGCCAC  
CP-2  
CP-3.10  
Y Q C F P E I S H L W G Q Y S P F F S L  
57  
GTTACCAATGTTTCCAGAAATTTCTCACTTGTGGGGTCAATACTCTCCATTCTTCTCTT  
121 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+  
180  
CAATGGTTACAAAGGGTCTTTAAAGAGTGAACACCCAGTTATGAGAGGTAAGAAGAGAA  
  
A D E S A I S P D V P K G C R V T F V Q  
77  
TGGCTGACGAATCTGCTATTTCTCCAGACGTTCCAAAGGGTGTAGAGTTACTTTCGTTT  
181 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+  
240  
ACCGACTGCTTAGACGATAAAGAGGTCTGCAAGGTTTCCCGACATCTCAATGAAAGCAAG  
CP-4.10  
CP-5.10  
V L S R H G A R Y P T S S K S K K Y S A  
97  
AAGTTTTGTCTAGACACGGTGCTAGATACCCAACTTCTTCTAAGTCTAAGAAGTACTCTG  
241 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+  
300  
TTCAAAACAGATCTGTGCCACGATCTATGGGTGAAGAAGATTCAGATTCTTCATGAGAC  
  
L I E A I Q K N A T A F K G K Y A F L K  
117  
CTTTGATTGAAGCTATTCAAAGAAGCTACTGCTTTCAAGGGTAAGTACGCTTTCTTGA  
301 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+  
360  
GAACTAACTTCGATAAGTTTCTTGGCATGACGAAAGTTCCCATTCATGCGAAAGAACT  
CP-6  
CP-7.10  
T Y N Y T L G A D D L T P F G E Q Q M V  
137  
AGACTTACAACCTACACTTTGGGTGCTGACGACTTGACTCCATTCCGGTGAACAACAAATGG  
361 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+  
420  
TCTGAATGTTGATGTGAAACCCACGACTGCTGAACTGAGGTAAGCCACTTGTGTTTACC  
  
N S G I K F Y R R Y K A L A R K I V P F  
157  
TTAACTCTGGTATTAAGTTCTACAGAAGATACAAGGCTTTGGCTAGAAAGATTGTTCCAT  
421 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+  
480  
AATTGAGACCATAATTCAAGATGTCTTCTATGTTCCGAAACCGATCTTTCTAACAAGGTA  
CP-8.10  
CP-9.10

V R A S G S D R V I A S A E K F I E G F  
177 TC GTT AGAG CT TCT GGT TCT GAC AGA GTT ATT GC TT CTG CT GA AA AG TT CAT TGA AG GTT  
481 -----+-----+-----+-----+-----+-----+-----+  
540 AG CAAT CTC GA AG ACCA AG ACT GT CT CA ATA AC GA AG AC GACT TTT CA AG TA ACT TCCA A  
  
Q S A K L A D P G A N P H Q A S P V I N  
197 TCC AAT CTG CTA AG TTGG CT GAC CCAG GTG CTA ACCC ACAC CA AG CT TCT CCAG TTATTA  
541 -----+-----+-----+-----+-----+-----+-----+  
600 AG GTT AG AC GATT CA ACCG ACT GGG TCC AC GATT GGG TGT GGT TCG AA AG GTT CA ATA AT  
CP-10.10  
CP-11.10  
V I I P E G A G Y N N T L D H G L C T A  
217 ACG TTATT ATT CCAGA AG GTG CT GGT TACA ACA AC ACT TTGG ACC AC GG TT TGT GT ACT G  
601 -----+-----+-----+-----+-----+-----+-----+  
660 TG CAATA ATA AG GTCTT CCAC GACCA AT GTT GTT GTG AA ACCT GGT GCC AA AC AT GAC  
  
F E E S E L G D D V E A N F T A V F A P  
237 CTTT CGA AGA AT CT GA ATT GGG T GAC GAC GTT GA AG CTA ACT TCA CTG CTG TTTT CG CT C  
661 -----+-----+-----+-----+-----+-----+-----+  
720 GAA AG CT TCT TAG ACTT AAC CC ACT GCT GCA ACT TCG ATT GA AGT GAC GAC AAA AG CG GAG  
CP-12.10  
  
P I R A R L E A H L P G V N L T D E D V  
257 CAC CTATT AG AG CTAG ATT GGA AG CT CACT TGCC AG GTG TTA ACT TG ACT GAC GA AG AC G  
721 -----+-----+-----+-----+-----+-----+-----+  
780 GT GGATA AT CT CG ATCTAAC CTTCG AGTGA AC GG TCC ACA ATT GA ACT GACT GCTT CTGC  
  
CP-13.10  
V N L M D M C P F D T V A R T S D A T Q  
277 TT GTTAA CT TGAT GG AC AT GT GTCC ATT CG AC ACT GTT GCT AGA ACT TCT GAC GCT ACT C  
781 -----+-----+-----+-----+-----+-----+-----+  
840 AACA ATT GA ACT AC CTGTAC AC AG GTA AG CTGTG ACA AC GAT CTTGA AG ACT GCG ATG AG  
  
L S P F C D L F T H D E W I Q Y D Y L Q  
297 AAT TGT CTCC ATT CTGT GACT GTT TCA CT CAC GAC GA ATGG ATT CA ATAC GACT ACTT GC  
841 -----+-----+-----+-----+-----+-----+-----+  
900 TTA ACAG AG GTAA GAC ACT GA ACA AGT GAG TGTG CT TAC CTA AG TTAT GCT GAT GA AC G  
CP-14.10  
CP-15.10  
S L G K Y Y G Y G A G N P L G P A Q G V  
317 AAT CT TTGG GTA AG TACT AC GG TTAC GG TGTG CTA ACC CAT TGG GTTCC AG CT CA AG GTG  
901 -----+-----+-----+-----+-----+-----+-----+  
960 TTAG AAAC CCATT CAT GATGCC AATGCC AC GACC ATTGG GTA ACCC AG GTT CG AG TTCC AC  
  
G F V N E L I A R L T H S P V Q D H T S  
337

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          TTGTTTCGTTAACGAATTGATTGCTAGATTGACTCACTCTCCAGTTCAAGACCACACTT
961 -----+-----+-----+-----+-----+-----+
1020 AACCAAAGCAATTGCTTAACTAACGATCTAACTGAGTGAGAGGTCAAGTTCTGGTGTGAA
          CP-16.10
          CP-17.10
          T N H T L D S N P A T F P L N A T L Y A
357 CTACTAACCACACTTTGGACTCTAACCCAGCTACTTTCCCATTTGAACGCTACTTTGTACG
1021 -----+-----+-----+-----+-----+
1080 GATGATTGGTGTGAAACCTGAGATTGGGTCGATGAAAGGGTAACTTGCGATGAAACATGC
          D F S H D N T M V S I F F A L G L Y N G
377 CTGACTTCTCTCAGACAACACTATGGTTTCTATTTTCTTCGCTTTGGGTTTGTACAACG
1081 -----+-----+-----+-----+-----+
1140 GACTGAAGAGAGTGCTGTTGTGATACCAAAGATAAAAGAAGCGAAACCCAAACATGTTGC
          CP-18.10
          CP-19.10
          T K P L S T T S V E S I E E T D G Y A A
397 GTACTAAGCCATTGTCTACTACTTCTGTTGAATCTATTGAAGAACTGACGGTTACGCTG
1141 -----+-----+-----+-----+-----+
1200 CATGATTCGGTAACAGATGATGAAGACAACCTTAGATAACTTCTTTGACTGCCAATGCGAC
          S W T V P F A A R A Y V E M M Q C E A E
417 CTTCTTGGACTGTTCCATTGCTGCTAGAGCTTACGTTGAAATGATGCAATGTGAAGCTG
1201 -----+-----+-----+-----+-----+
1260 GAAGAACCTGACAAGGTAAGCCACGATCTCGAATGCAACTTTACTACGTTACACTTCGAC
          CP-20.10
          CP-21.10
          K E P L V R V L V N D R V V P L H G C G
437 AAAAGGAACCATTGGTTAGAGTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTG
1261 -----+-----+-----+-----+-----+
1320 TTTTCCTTGGTAACCAATCTCAAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACAC
          V D K L G R C K R D D F V E G L S F A R
457 GTGTTGACAAGTTGGGTAGATGTAAGAGAGACGACTTCGTTGAAGGTTTGTCTTTTCGCTA
1321 -----+-----+-----+-----+-----+
1380 CACAACTGTTCAACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGAT
          CP-22.10
          S G G N W E E C F A * Eco RI 467
          GATCTGGTGGTAACTGGGAAGAATGTTTCGCTTAAGAATTCATATA
1381 -----+-----+-----+-----+-----+ 1426
          CTAGACCACCATTGACCCTTCTTACAAAGCGAATTCTTAAGTATAT

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Figure 18

	1
50	
<i>P. involutus</i> (phyA1)	----- ~FPipeseqR nWSPYSPYFP LAEyKA....
pPaGCQInqV	
<i>P. involutus</i> (phyA2)	----- ~FsipeseqR nWSPYSPYFP LAEyKA....
pPaGCeInqV	
<i>T. pubescens</i>	----- ~LDvtRDVqQ sWSmYSPYFP aAtyvA....
pPaSCQInqV	
<i>A. pediades</i>	----- ~pffpPQIQd sWAaYTPYYP VqAyTP....
ppKDCKITqV	
<i>P. lycii</i>	----- ~LPipAQnTs nWGPYdPFFP VEpyAA....
pPEGCTVTqV	
<i>A. terreus</i> 9a1	KhSDcNSVDh GYQCfPELSH kWGLYAPYFS LqDESPPFLD
VPEDCHITFV	
<i>A. terreus</i> cbs	NhSDcTSVDr GYQCfPELSH kWGLYAPYFS LqDESPPFLD
VPDDCHITFV	
<i>A. niger</i> var. <i>awamori</i>	NqSTCDTVDq GYQCfSETSH LWGQYAPFFS LANESAISPD
VPaGCRVTFa	
<i>A. niger</i> T213	NqSSCDTVDq GYQCfSETSH LWGQYAPFFS LANESVISPD
VPaGCRVTFa	
<i>A. niger</i> NRRL3135	NqSSCDTVDq GYQCfSETSH LWGQYAPFFS LANESVISPE
VPaGCRVTFa	
<i>A. fumigatus</i> ATCC13073	GSKSCDTVDl GYQCsPATSH LWGQYSPFFS LEDElSVSSK
LPKDCRITLV	
<i>A. fumigatus</i> ATCC32722	GSKSCDTVDl GYQCsPATSH LWGQYSPFFS LEDElSVSSK
LPKDCRITLV	
<i>A. fumigatus</i> ATCC58128	GSKSCDTVDl GYQCsPATSH LWGQYSPFFS LEDElSVSSK
LPKDCRITLV	
<i>A. fumigatus</i> ATCC26906	GSKSCDTVDl GYQCsPATSH LWGQYSPFFS LEDElSVSSK
LPKDCRITLV	
<i>A. fumigatus</i> ATCC32239	GSKACDTVEl GYQCsPGtSH LWGQYSPFFS LEDElSVSSD
LPKDCRVTFV	
<i>E. nidulans</i>	QNHSCNTaDg GYQCfPNVSH VWGQYSPYFS IEQESAISEd
VPhGCeVTFV	
<i>T. thermophilus</i>	DSHSCNTVEg GYQCrPEISH SWGQYSPFFS LADQSEISPD
VPQNCKITFV	
<i>T. lanuginosa</i>	----- ~nvDIAR hWGQYSPFFS LAEvSEISPA
VPKGCReVfV	
<i>M. thermophila</i>	ESRPCDTpDl GFQCgTAISH FWGQYSPYFS VPSElDaS..
IPDDCeVTFa	
Consensus Seq. 11	NSHSCDTVD- GYQC-PEISH LWGQYSPFFS LADESAISPD
VPKGCReVTFV	
100	
<i>P. involutus</i> (phyA1)	NIIqRHGARF PTSGaTtRik AgLtKLQgvq nftDAKFnFI
KSPKYdLGns	
<i>P. involutus</i> (phyA2)	NIIqRHGARF PTSGaAtRik AgLsKLQsvq nftDPKFDFI
KSFTYdLGts	
<i>T. pubescens</i>	HIIqRHGARF PTSGaAKRiq TaVAKLKaaS nytDPILAFV
tnYtYSLGqD	
<i>A. pediades</i>	NIIqRHGARF PTSGaGtRiq AaVKKLQsak TytdPRLDfL
tnYtYTLGhD	
<i>P. lycii</i>	NLIqRHGARW PTSGarsRqv AaVAKIQmar PftDPKYEFL
NdFvYkFGvA	
<i>A. terreus</i> 9a1	QVLARHGARS PThSKTKaYA AtIAaIQKSA TaFpGKYAFL
QSYNYSLDSE	
<i>A. terreus</i> cbs	QVLARHGARS PTdSKTKaYA AtIAaIQKNA TaLpGKYAFL
KSYNYSMGSE	
51	

<i>A. niger</i> var. <i>awamori</i>	QVLSRHGARY PTeSKGKKYS ALIEeIQQNv TtFDGKYAFL
KTYNYSLGAD	
<i>A. niger</i> T213	QVLSRHGARY PTeSKGKKYS ALIEeIQQNv TtFDGKYAFL
KTYNYSLGAD	
<i>A. niger</i> NRRL3135	QVLSRHGARY PTdSKGKKYS ALIEeIQQNA TtFDGKYAFL
KTYNYSLGAD	
<i>A. fumigatus</i> ATCC13073	QVLSRHGARY PTSSKSsKKYk kLVtaIQaNA TdFKGKFAFL
KTYNYTLGAD	
<i>A. fumigatus</i> ATCC32722	QVLSRHGARY PTSSKSsKKYk kLVtaIQaNA TdFKGKFAFL
KTYNYTLGAD	
<i>A. fumigatus</i> ATCC58128	QVLSRHGARY PTSSKSsKKYk kLVtaIQaNA TdFKGKFAFL
KTYNYTLGAD	
<i>A. fumigatus</i> ATCC26906	QVLSRHGARY PTSSKSsKKYk kLVtaIQaNA TdFKGKFAFL
KTYNYTLGAD	
<i>A. fumigatus</i> ATCC32239	QVLSRHGARY PTASKSsKKYk kLVtaIQKNA TeFKGKFAFL
ETNYNYTLGAD	
<i>E. nidulans</i>	QVLSRHGARY PTeSKSKaYS GLIEaIQKNA TsFwGQYAFL
ESYNYTLGAD	
<i>T. thermophilus</i>	QLLSRHGARY PTSSKTELYS qLIrRIQKtA TaYKGyYAFL
KdYrYqLGAN	
<i>T. lanuginosa</i>	QVLSRHGARY PTAhKSEvYA ELLQRIQDta TeFKGDFaFL
RdYaYhLGAD	
<i>M. thermophila</i>	QVLSRHGARA PtlkRAasYv DLIDRIHhGA isYgPgYEFL
RTYDYTLGAD	
Consensus Seq. 11	QVLSRHGARY PTSSKSsKKYS ALIERIQKNA T-FKGKYAFL
KTYNYTLGAD	

101

150

<i>P. involutus</i> (phyA1)	DLvPFGAAQs fDAGqEaFaR YskLvSKNnL PFIRadGSDR
VVDSAtNWtA	
<i>P. involutus</i> (phyA2)	DLvPFGAAQs fDAGLEvFaR YskLvSsDnL PFIRsdGSDR
VVDtAtNWtA	
<i>T. pubescens</i>	sLveLGatQs sEAGqEaFtR YsSLvSaDeL PFVRASGSDR
VVATANNWtA	
<i>A. pediades</i>	DLvPFGAlQs sQAGeEtFQR YsflvSKEnL PFVRASSsNR
VVDSAtNWtE	
<i>P. lycii</i>	DLlPFGANQs hQTGtDMYtR YsTLfEgGdV PFVRAAGdQR
VVDSStNWtA	
<i>A. terreus</i> 9a1	ELTPFGGrNQL rDlGaQFYeR YNAL.TRHIn PFVRATDAsR
VhESAeKFVE	
<i>A. terreus</i> cbs	NLTPFGGrNQL qDlGaQFYRR YDTL.TRHIn PFVRAADsSR
VhESAeKFVE	
<i>A. niger</i> var. <i>awamori</i>	DLTPFGEQEL VNSGIKFYQR YESL.TRNII PFIRSSGSsR
VIASGEKFIE	
<i>A. niger</i> T213	DLTPFGEQEL VNSGIKFYQR YESL.TRNII PFIRSSGSsR
VIASGEKFIE	
<i>A. niger</i> NRRL3135	DLTPFGEQEL VNSGIKFYQR YESL.TRNIV PFIRSSGSsR
VIASGKKFIE	
<i>A. fumigatus</i> ATCC13073	DLTPFGEQQL VNSGIKFYQR YKAL.ARSVV PFIRASGSDR
VIASGEKFIE	
<i>A. fumigatus</i> ATCC32722	DLTPFGEQQL VNSGIKFYQR YKAL.ARSVV PFIRASGSDR
VIASGEKFIE	
<i>A. fumigatus</i> ATCC58128	DLTPFGEQQL VNSGIKFYQR YKAL.ARSVV PFIRASGSDR
VIASGEKFIE	
<i>A. fumigatus</i> ATCC26906	DLTAFGEQQL VNSGIKFYQR YKAL.ARSVV PFIRASGSDR
VIASGEKFIE	
<i>A. fumigatus</i> ATCC32239	DLTPFGEQQM VNSGIKFYQK YKAL.AgSVV PFIRSSGSsR
VIASGEKFIE	
<i>E. nidulans</i>	DLTifGENQM VDsgaKFYRR YKnL.ARKnt PFIRASGSDR
VVASAEKFIn	

<i>T. thermophilus</i>	DLTPFGENQM IQLGIKFYnH YKSL.ARNv PFVRCSGSDR
VIASGr1FIE	
<i>T. lanuginosa</i>	NLTRFGEEQM MESGrQFYHR YREq.AREIV PFVRAAGSAR
VIASAEfFnr	
<i>M. thermophila</i>	ELTRtGQQQM VNSGIKFYRR YRAL.ARKsI PFVRTAGQDR
VVhSAENFtQ	
Consensus Seq. 11	DLTPFGENQM VNSGIKFYRR YKAL-ARNIV PFVRASGSDR
VIASAEKFIE	
151	
200	
<i>P. involutus</i> (phyA1)	GFaSA..... ..shNtvqPk LNLILPQ..T gNDTLEDNMC
PAaGD.....	
<i>P. involutus</i> (phyA2)	GFaSA..... ..srNaiqPk LDLILPQ..T gNDTLEDNMC
PAaGE.....	
<i>T. pubescens</i>	GFaIA..... ..ssNsITPV LSVIISE..A gNDTLDDNMC
PAaGD.....	
<i>A. pediacdes</i>	GFsAA..... ..shHvlnPI LfVILSE..S LNDTLDDAMC
PnaGs.....	
<i>P. lycii</i>	GFgdA..... ..sgEtvlPt LQVVLQE..E gNcTLcNNMC
PnevD.....	
<i>A. terreus</i> 9a1	GFQTARqDdh hAnpHQPSPr VDVaIPEGSA YNNTLEHSLC
TAFES...ST	
<i>A. terreus</i> cbs	GFQNARqGDP hAnpHQPSPr VDVVIPEGTA YNNTLEHSIC
TAFEa...ST	
<i>A. niger</i> var. <i>awamori</i>	GFQSTKLkDP rAqpgQSSPk IDVVISeASS sNNTLDpGtC
TvFED...Se	
<i>A. niger</i> T213	GFQSTKLkDP rAqpgQSSPk IDVVISeASS sNNTLDpGtC
TvFED...Se	
<i>A. niger</i> NRRL3135	GFQSTKLkDP rAqpgQSSPk IDVVISeASS sNNTLDpGtC
TvFED...Se	
<i>A. fumigatus</i> ATCC13073	GFQqAKLADP gAt.NRAAPa ISVIIPeSeT FNNTLDHGVC
TkFEa...Sq	
<i>A. fumigatus</i> ATCC32722	GFQqAKLADP gAt.NRAAPa ISVIIPeSeT FNNTLDHGVC
TkFEa...Sq	
<i>A. fumigatus</i> ATCC58128	GFQqAKLADP gAt.NRAAPa ISVIIPeSeT FNNTLDHGVC
TkFEa...Sq	
<i>A. fumigatus</i> ATCC26906	GFQqAKLADP gAt.NRAAPa ISVIIPeSeT FNNTLDHGVC
TkFEa...Sq	
<i>A. fumigatus</i> ATCC32239	GFQqANVADP gAt.NRAAPV ISVIIPeSeT YNNTLDHSVC
TnFEa...Se	
<i>E. nidulans</i>	GFRkaQLhDh g.s.gQATPV VNVIIPeIdG FNNTLDHStC
vSFEN...de	
<i>T. thermophilus</i>	GFQSAKVlDP hSdKHDApPt INVIIeEGPS YNNTLDtGSc
PvFED...SS	
<i>T. lanuginosa</i>	GFQdAKdrDP rSnKDQaEPV INVIISeETG sNNTLDgltC
PAaEE...AP	
<i>M. thermophila</i>	GFHSA1LADR gStvRPTlPy dmVVIPETAG aNNTLHNDLC
TAFEEgpyST	
Consensus Seq. 11	GFQSAKLADP -A--HQASPV INVIIPEGSG YNNTLDHGLC
TAFED---ST	
201	
250	
<i>P. involutus</i> (phyA1)	.SDpqvnaWl AVafPSItAR LNAaapSVNL TDtDafNLVs
LCAFlTVSK.	
<i>P. involutus</i> (phyA2)	.SDpqvDaWl AsafPSVtAQ LNAaapGaNL TDADafNLVs
LCPFmTVSK.	
<i>T. pubescens</i>	.SDpqvnQWl AqFAPPMtAR LNAgaPGaNL TDtDtyNLLt
LCPFETVat.	

<i>A. pediades</i>	.SDpqtGiWT SIYGTPIanR LNqqaPGaNI TAADVsnLIp
LCAFETIVK.	
<i>P. lycii</i>	.GDESt.twl GVFApNIaR LNAAApsaNL SDsDaLtLMD
MCPFDTLSS.	
<i>A. terreus</i> 9a1	VGDDAVANFT AVFAPAIaQR LEAdLPGVQL StDDVVNLMA
MCPFETVSI	
<i>A. terreus</i> cbs	VGDAADNFT AVFAPAIaKR LEAdLPGVQL SADDVVNLMA
MCPFETVSI	
<i>A. niger</i> var. <i>awamori</i>	LADtveANFT AtFAPSIRqR LEndLSGVtL TdEVtyLMD
MCSFDTISTs	
<i>A. niger</i> T213	LADtveANFT AtFAPSIRqR LEndLSGVtL TdEVtyLMD
MCSFDTISTs	
<i>A. niger</i> NRRL3135	LADtveANFT AtFvPSIRqR LEndLSGVtL TdEVtyLMD
MCSFDTISTs	
<i>A. fumigatus</i> ATCC13073	LGDEVAANFT ALFAPdIRAR aEkhlPGVtL TDEDVVSLMD
MCSFDTVART	
<i>A. fumigatus</i> ATCC32722	LGDEVAANFT ALFAPdIRAR aEkhlPGVtL TDEDVVSLMD
MCSFDTVART	
<i>A. fumigatus</i> ATCC58128	LGDEVAANFT ALFAPdIRAR aEkhlPGVtL TDEDVVSLMD
MCSFDTVART	
<i>A. fumigatus</i> ATCC26906	LGDEVAANFT ALFAPdIRAR aKkhLPGVtL TDEDVVSLMD
MCSFDTVART	
<i>A. fumigatus</i> ATCC32239	LGDEVEANFT ALFAPAIRAR IEkhLPGVQL TDDDVVSLMD
MCSFDTVART	
<i>E. nidulans</i>	rADEiEANFT AIMGPPIRKR LEndLPGIKL TNENViyLMD
MCSFDTMART	
<i>T. thermophilus</i>	gGHDAQEKFA kqFAPAILEK IKDhLPGVDL AvsDVpyLMD
LCPFETLARN	
<i>T. lanuginosa</i>	.DptqpAEf1 qVFGPRVlKk ItkhMPGVNL TLEDVplFMD
LCPFDTVGSd	
<i>M. thermophila</i>	IGDDAQDtyl StFAGPItAR VNAnLPGaNL TDADtValMD
LCPFETVAsS	
Consensus Seq. 11	LGDDAEANFT AVFAPPiRAR LEA-LPGVNL TDEDVVNLMD
MCPFDTVART	
300	251
<i>P. involutus</i> (phyA1)	..... .ekksdF CtLFegiPGs FeaFAYggdL
dKfYgTgYgQ	
<i>P. involutus</i> (phyA2)	..... .eqksdF CtLFegiPGs FeaFAYagdL
dKfYgTgYgQ	
<i>T. pubescens</i>	..... .errSeF CDiYeelqAE .daFAYnadL
dKfYgTgYgQ	
<i>A. pediades</i>	..... .etpSPF CNLF..TPEE FaQFEYfgdL
dKfYgTgYgQ	
<i>P. lycii</i>	..... .gnaSPF CDLF..TAAE YvsYEYYdL
dKYyGtGPGN	
<i>A. terreus</i> 9a1	dD..Aht... ..LSPF CDLF..TatE WtQYNYLlSL
dKYyGYGGGN	
<i>A. terreus</i> cbs	dD..Aht... ..LSPF CDLF..TAAE WtQYNYLlSL
dKYyGYGGGN	
<i>A. niger</i> var. <i>awamori</i>	Tv..DTK... ..LSPF CDLF..ThDE WiHYDYlQSL
kKYyGHGAGN	
<i>A. niger</i> T213	Tv..DTK... ..LSPF CDLF..ThDE WiHYDYlRSL
kKYyGHGAGN	
<i>A. niger</i> NRRL3135	Tv..DTK... ..LSPF CDLF..ThDE WiNYDYlQSL
kKYyGHGAGN	
<i>A. fumigatus</i> ATCC13073	SD..ASQ... ..LSPF CQLF..ThNE WkKYNYlQSL
gKYyGYGAGN	
<i>A. fumigatus</i> ATCC32722	SD..ASQ... ..LSPF CQLF..ThNE WkKYNYlQSL
gKYyGYGAGN	

<i>A. fumigatus</i> ATCC58128	SD..ASQ... ..LSPF CQLF..ThNE WkKYNYLQSL
gKYYGYGAGN	
<i>A. fumigatus</i> ATCC26906	SD..ASQ... ..LSPF CQLF..ThNE WkKYNYLQSL
gKYYGYGAGN	
<i>A. fumigatus</i> ATCC32239	AD..ASE... ..LSPF CAIF..ThNE WkKYDYLQSL
gKYYGYGAGN	
<i>E. nidulans</i>	AH..GTE... ..LSPF CAIF..TEKE WlQYDYLQSL
sKYYGYGAGS	
<i>T. thermophilus</i>	ht..DT.... ..LSPF CALs..TqEE WqayDYyQSL
gKYYGnGGGN	
<i>T. lanuginosa</i>	PvlfPrQ... ..LSPF CHLF..TADD WmaYDYyTL
dKYYSHGGGS	
<i>M. thermophila</i>	SsdpATadag ggnggrpLSPF CrLF..SEsE WrayDYLQSV
gKWYGYGPGN	

Consensus Seq. 11	SD--ATQ--- -----LSPF CDLF--TADE W-QYDYLQSL -
KYYGYGAGN	

301

350	
<i>P. involutus</i> (phyA1)	eLGPvQGVGY vNELIARLTN S.AVRDNTqT NRTLDA SPVT
FPLNkTFYAD	
<i>P. involutus</i> (phyA2)	ALGPvQGVGY iNELLARLTN S.AVNDNTqT NRTLDAApDT
FPLNkTMYAD	
<i>T. pubescens</i>	PLGPvQGVGY iNELIARLTa q.nVsDHTqT NsTLDS SPET
FPLNrTLYAD	
<i>A. pediades</i>	PLGPvQGVGY iNELLARLT E m.PVRDNTqT NRTLDS SP1T
FPLDrSIYAD	
<i>P. lycii</i>	ALGPvQGVGY vNELLARLTg q.AVRDETqT NRTLDS DPAT
FPLNrTFYAD	
<i>A. terreus</i> 9a1	PLGPvQGVGW aNELMARLTR A.PVHDHTCv NNTLDAS PAT
FPLNATLYAD	
<i>A. terreus</i> cbs	PLGPvQGVGW aNELIARLTR S.PVHDHTCv NNTLDANPAT
FPLNATLYAD	
<i>A. niger</i> var. <i>awamori</i>	PLGPTQGVGY aNELIARLTH S.PVHDDTSS NHTLDSNPAT
FPLNSTLYAD	
<i>A. niger</i> T213	PLGPTQGVGY aNELIARLTH S.PVHDDTSS NHTLDSNPAT
FPLNSTLYAD	
<i>A. niger</i> NRRL3135	PLGPTQGVGY aNELIARLTH S.PVHDDTSS NHTLDS SPAT
FPLNSTLYAD	
<i>A. fumigatus</i> ATCC13073	PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT
FPLNATMYvD	
<i>A. fumigatus</i> ATCC32722	PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT
FPLNATMYvD	
<i>A. fumigatus</i> ATCC58128	PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT
FPLNATMYvD	
<i>A. fumigatus</i> ATCC26906	PLGPAQGIGF tNELIARLTR S.PVQDHTST NsTLvSNPAT
FPLNATMYvD	
<i>A. fumigatus</i> ATCC32239	PLGPAQGIGF tNELIARLTN S.PVQDHTST NsTLDS DPAT
FPLNATIYvD	
<i>E. nidulans</i>	PLGPAQGIGF tNELIARLTQ S.PVQDNTST NHTLDSNPAT
FPLDrkLYAD	
<i>T. thermophilus</i>	PLGPAQGVGF vNELIARMTH S.PVQDYTTV NHTLDSNPAT
FPLNATLYAD	
<i>T. lanuginosa</i>	AFGPSRGVGF vNELIARMTg N1PVKDHTTV NHTLDdNPET
FPLDAVLYAD	
<i>M. thermophila</i>	PLGPTQGVGF vNELLARLA. GvPVRDgTST NRTLGDGP rT
FPLGrPLYAD	

Consensus Seq. 11	PLGPAQGVGF -NELIARLTH S-PVQDHTST NHTLDSNPAT
FPLNATLYAD	



351

400  
*P. involutus* (phyA1) FSHDNlMVAV FsAMGLFrqP aPLSTSvpNP wrt.....Wr  
 TSSlVPFSGR  
*P. involutus* (phyA2) FSHDNlMVAV FsAMGLFrqS aPLSTSTpDP nrt.....Wl  
 TSSvVPFSAR  
*T. pubescens* FSHDNqMVAI FsAMGLFNqS aPLdPTTpDP art.....Fl  
 vkkiVPFSAR  
*A. pediades* LSHDNqMIAI FsAMGLFNqS sPLdPSfpNP krt.....Wv  
 TSRLtPFSAR  
*P. lycii* FSHDNTMVPI FaALGLFNAT a.LdPlkpDe nrl.....Wv  
 DSKlVPFSGH  
*A. terreus* 9a1 FSHDSnLVSI FWALGLYNGT aPLSQtSVES Vs..QTDGYA  
 AAWTVPFAAR  
*A. terreus* cbs FSHDSnLVSI FWALGLYNGT KPLSQtTVEd It..rTDGYA  
 AAWTVPFAAR  
*A. niger* var. *awamori* FSHDNGIISI LFALGLYNGT KPLSTTTVEN It..QTDGFS  
 SAWTVPFASR  
*A. niger* T213 FSHDNGIISI LFALGLYNGT KPLSTTTVEN It..QTDGFS  
 SAWTVPFASR  
*A. niger* NRRL3135 FSHDNGIISI LFALGLYNGT KPLSTTTVEN It..QTDGFS  
 SAWTVPFASR  
*A. fumigatus* ATCC13073 FSHDNSMVSI FFALGLYNGT EPLSrTSVES ak..EldGYS  
 ASWvVPFGAR  
*A. fumigatus* ATCC32722 FSHDNSMVSI FFALGLYNGT gPLSrTSVES ak..EldGYS  
 ASWvVPFGAR  
*A. fumigatus* ATCC58128 FSHDNSMVSI FFALGLYNGT EPLSrTSVES ak..EldGYS  
 ASWvVPFGAR  
*A. fumigatus* ATCC26906 FSHDNSMVSI FFALGLYNGT EPLSrTSVES ak..EldGYS  
 ASWvVPFGAR  
*A. fumigatus* ATCC32239 FSHDNGMIPI FFAMGLYNGT EPLSQtSeES tk..ESNGYS  
 ASWAVPFGAR  
*E. nidulans* FSHDNSMISI FFAMGLYNGT QPLSmdSVES Iq..EmDGYA  
 ASWTVPFGAR  
*T. thermophilus* FSHDNTMtSI FaALGLYNGT akLSTTeIKS Ie..ETDGYS  
 AAWTVPFGGR  
*T. lanuginosa* FSHDNTMtGI FsAMGLYNGT KPLSTSkIQP ptgaAADGYA  
 ASWTVPFAAR  
*M. thermophila* FSHDNdMMGV LgALGaYDgV pPLdkTArrd ..peElGGYA  
 ASWAVPFAAR  
 Consensus Seq. 11 FSHDNTMVSI FFALGLYNGT KPLSTTSVES I---ETDGYA  
 ASWTVPFAAR

401

450  
*P. involutus* (phyA1) mvVerLsC.. fGt.....Tk VRVLVQDQVq  
 PLEfCGgDRn  
*P. involutus* (phyA2) maVerLsC.. AGt.....Tk VRVLVQDQVq  
 PLEfCGgDQd  
*T. pubescens* mvVerLDC.. GGa.....Qs VRLLVNDaVq  
 PLafCGaDts  
*A. pediades* mvtErLlCQr DgtGsGGpsr imrNgnvQTF VRILVNDaLq  
 PLkfCGgDmd  
*P. lycii* mtVEkLaC.. .....sgKea VRVLVNDaVq  
 PLEfCGg.vd  
*A. terreus* 9a1 AYVEMMQCrA .....EK...EPL VRVLVNDRVM  
 PLHGCPtDKL  
*A. terreus* cbs AYIEMMQCrA .....EK...QPL VRVLVNDRVM  
 PLHGCAVDNL  
*A. niger* var. *awamori* lYVEMMQCQA .....EQ...EPL VRVLVNDRVV  
 PLHGCPIDaL

<i>A. niger</i> T213	1YVEMMQCQA .....	..EQ...EPL VRVLVNDRVV
PLHGCPIDaL		
<i>A. niger</i> NRRL3135	1YVEMMQCQA .....	..EQ...EPL VRVLVNDRVV
PLHGCPVDaL		
<i>A. fumigatus</i> ATCC13073	AYfEtMQCKS .....	..EK...EPL VRaLINDRVV
PLHGCDVDKL		
<i>A. fumigatus</i> ATCC32722	AYfEtMQCKS .....	..EK...EPL VRaLINDRVV
PLHGCDVDKL		
<i>A. fumigatus</i> ATCC58128	AYfEtMQCKS .....	..EK...ESL VRaLINDRVV
PLHGCDVDKL		
<i>A. fumigatus</i> ATCC26906	AYfEtMQCKS .....	..EK...EPL VRaLINDRVV
PLHGCDVDKL		
<i>A. fumigatus</i> ATCC32239	AYfEtMQCKS .....	..EK...EPL VRaLINDRVV
PLHGCAVDKL		
<i>E. nidulans</i>	AYfELMQCE. ....	..KK...EPL VRVLVNDRVV
PLHGCAVDKF		
<i>T. thermophilus</i>	AYIEMMQCDD .....	..sD...EPV VRVLVNDRVV
PLHGCEVDsL		
<i>T. lanuginosa</i>	AYVELLRcET ETsSeEEeEG ..	..ED...EPF VRVLVNDRVV
PLHGCrVDRW		
<i>M. thermophila</i>	iYVEkMRCSG GGgGgGGgEG ..	..rQekdEem VRVLVNDRVV
TLkGCGaDER		
Consensus Seq. 11	AYVEMMQCEA GG-G-GG-EG --	..EK---EPL VRVLVNDRVV
PLHGCGVDKL		

	451		482
<i>P. involutus</i> (phyA1)	GlCtLAKFVE	SqTFARSDga	GDFEKCFats a~
<i>P. involutus</i> (phyA2)	GlCaLDKFVE	SqAYARSGga	GDFEKCLatt v~
<i>T. pubescens</i>	GvCtLDAFVE	SqAYARNDge	GDFEKCFat~ --
<i>A. pediades</i>	SlCtLEAFVE	SqkYARedgq	GDFEKCFD~ --
<i>P. lycii</i>	GvCELSAFVE	SqTYAReNgq	GDFAKCgfv se
<i>A. terreus</i> 9a1	GRCKrDAFVA	GLSFAQAG..	GNWADCF~~~ --
<i>A. terreus</i> cbs	GRCKrDDFVE	GLSFARAG..	GNWAECF~~~ --
<i>A. niger</i> var. <i>awamori</i>	GRCtrDsFVr	GLSFARSG..	GDWAECsA~~ --
<i>A. niger</i> T213	GRCtrDsFVr	GLSFARSG..	GDWAECFA~~ --
<i>A. niger</i> NRRL3135	GRCtrDsFVr	GLSFARSG..	GDWAECFA~~ --
<i>A. fumigatus</i> ATCC13073	GRCKLNDFVK	GLSWARSG..	GNWGECSF~~ --
<i>A. fumigatus</i> ATCC32722	GRCKLNDFVK	GLSWARSG..	GNWGECSF~~ --
<i>A. fumigatus</i> ATCC58128	GRCKLNDFVK	GLSWARSG..	GNWGECSF~~ --
<i>A. fumigatus</i> ATCC26906	GRCKLNDFVK	GLSWARSG..	GNWGECSF~~ --
<i>A. fumigatus</i> ATCC32239	GRCKLKDFVK	GLSWARSG..	GNSEQSFS~~ --
<i>E. nidulans</i>	GRCTLDDWVE	GLNFARSG..	GNWktCFT1~ --
<i>T. thermophilus</i>	GRCKrDDFVr	GLSFARqG..	GNWEGCYAas e~
<i>T. lanuginosa</i>	GRCRrDEWIK	GLTFARqG..	GHWDrcF~~~ --
<i>M. thermophila</i>	GmCtLErFIE	SMAFARGN..	GKWDlCFA~~ --
Consensus Seq. 11	GRCKLDDFVE	GLSFARSG--	GNWAECFA-- --

Figure 19

M G V F V V L L S I A T L F G S T S G T  
 20 ATGGGCGTGTTCGTGCTACTGTCCATTGCCACCTTGTTCGGTTCACATCCGGTACC  
 1 -----+-----+-----+-----+-----+-----+-----+-----  
 60 TACCCGCACAAGCAGCACGATGACAGGTAACGGTGAACAAGCCAAGGTGTAGGCCATGG  
 A L G P R G N S H S C D T V D G G Y Q C  
 40 GCCTTGGGTCTCGTGGTAATTCTCACTCTTGTGACACTGTTGACGGTGGTTACCAATGT  
 61 -----+-----+-----+-----+-----+-----+-----+-----  
 120 CGGAACCCAGGAGCACCATTAAGAGTGAGAACACTGTGACAACCTGCCACCAATGGTTACA  
 F P E I S H L W G T Y S P Y F S L A D E  
 60 TTCCAGAAATTTCTCACTTGTGGGTACCTACTCTCCATACTTCTCTTTGGCAGACGAA  
 121 -----+-----+-----+-----+-----+-----+-----+-----  
 180 AAGGCTCTTTAAAGAGTGAACACCCCATGGATGAGAGGTATGAAGAGAAACCGTCTGCCT  
 S A I S P D V P D D C R V T F V Q V L S  
 80 TCTGCTATTTCTCCAGACGTTCCAGACGACTGTAGAGTTACTTTTCGTTCAAGTTTGTCT  
 187 -----+-----+-----+-----+-----+-----+-----+-----  
 240 AGACGATAAAGAGGTCTGCAAGGTCTGCTGACATCTCAATGAAAGCAAGTTCAAAACAGA  
 R H G A R Y P T S S A S K A Y S A L I E  
 100 AGACACGGTGCTAGATACCCAACCTCTTCTGCGTCTAAGGCTTACTCTGCTTTGATTGAA  
 241 -----+-----+-----+-----+-----+-----+-----+-----  
 300 TCTGTGCCACGATCTATGGGTTGAAGAAGACGCAGATTCCGAATGAGACGAAACTAACTT  
 A I Q K N A T A F K G K Y A F L K T Y N  
 120 GCTATTCAAAGAACGCTACTGCTTTCAAGGGTAAGTACGCTTTCTTGAAGACTTACAAC  
 301 -----+-----+-----+-----+-----+-----+-----+-----  
 360 CGATAAGTTTCTTGCATGACGAAAGTTCCCATTCATGCGAAAGAAGTTCTGAATGTTG  
 Y T L G A D D L T P F G E N Q M V N S G  
 140 TACACTTTGGGTGCTGACGACTTGACTCCATTTCGGTGAAAACCAAATGGTTAACTCTGGT  
 361 -----+-----+-----+-----+-----+-----+-----+-----  
 420 ATGTGAAACCCACGACTGCTGAAGTGAAGGTAAGCCACTTTTGGTTTACCAATGAGACCA  
 I K F Y R R Y K A L A R K I V P F I R A  
 160 ATTAAGTTCTACAGAAGATACAAGGCTTTGGCTAGAAAGATTGTTCCATTCTAGAGCT  
 421 -----+-----+-----+-----+-----+-----+-----+-----  
 480 TAATTCAAGATGTCTTCTATGTTCCGAAACCGATCTTTCTAACAAGGTAAGTAATCTCGA  
 S G S D R V I A S A E K F I E G F Q S A  
 180 TCTGGTTCTGACAGAGTTATTGCTTCTGCTGAAAAGTTCATTGAAGGTTTCCAATCTGCT

481 -----+-----+-----+-----+-----+-----+-----  
 540 AGACCAAGACTGTCTCAATAACGAAGACGACTTTTCAAGTAACTTCCAAAGGTTAGACGA  
 K L A D P G S Q P H Q A S P V I N V I I  
 200 AAGTTGGCTGACCCAGGTTCTCAACCACACCAAGCTTCTCCAGTTATTAACGTGATCATT  
 541 -----+-----+-----+-----+-----+-----+-----  
 600 TTCAACCGACTGGGTCCAAGAGTTGGTGTGGTTCGAAGAGGTCAATAATTGCACTAGTAA  
 P E G S G Y N N T L D H G T C T A F E D  
 220 CCAGAAGGATCCGGTTACAACAACACTTTGGACCACGGTACTTGTACTGCTTTTGAAGAC  
 601 -----+-----+-----+-----+-----+-----+-----  
 660 GGTCTTCCTAGGCCAATGTTGTTGTGAAACCTGGTGCCATGAACATGACGAAAGCTTCTG  
 S E L G D D V E A N F T A L F A P A I R  
 240 TCTGAATTAGGTGACGACGTTGAAGCTAACTTCACTGCTTTGTTGCTCCAGCTATTAGA  
 661 -----+-----+-----+-----+-----+-----+-----  
 720 AGACTTAATCCACTGCTGCAACTTCGATTGAAGTGACGAAACAAGCGAGGTGATAATCT  
 A R L E A D L P G V T L T D E D V V Y L  
 260 GCTAGATTGGAAGCTGACTTGCCAGGTGTTACTTTGACTGACGAAGACGTTGTTTACTTG  
 721 -----+-----+-----+-----+-----+-----+-----  
 780 CGATCTAACCTTCGACTGAACGGTCCACAATGAACTGACTGCTTCTGCAACAAATGAAC  
 M D M C P F D T V A R T S D A T E L S P  
 280 ATGGACATGTGTCCATTGCACTGTGCTAGAACTTCTGACGCTACTGAATTGTCTCCA  
 781 -----+-----+-----+-----+-----+-----+-----  
 840 TACCTGTACACAGGTAAGCTGTGACAGCGATCTTGAAGACTGCGATGACTTAACAGAGGT  
 F C A L F T H D E W I Q Y D Y L Q S L G  
 300 TTCTGTGCTTTGTTCACTCACGACGAATGGATCCAATACGACTACTTGCAAAGCTTGGGT  
 841 -----+-----+-----+-----+-----+-----+-----  
 900 AAGACACGAAACAAGTGAGTGCTGCTTACCTAGGTTATGCTGATGAACGTTTTCGAACCCA  
 K Y Y G Y G A G N P L G P A Q G V G F A  
 320 AAGTACTACGGTTACGGTGCTGGTAACCCATTGGGTCCAGCTCAAGGTGTTGGTTTCGCT  
 901 -----+-----+-----+-----+-----+-----+-----  
 960 TTCATGATGCCAATGCCACGACCATTGGGTAAACCCAGGTGAGTTCACACAACCAAGCGA  
 N E L I A R L T H S P V Q D H T S T N H  
 340 AACGAATTGATTGCTAGATTGACTCACTCTCCAGTTCAAGACCACACTTCTACTAACCAC  
 961 -----+-----+-----+-----+-----+-----+-----  
 1020 TTGCTTAACTAACGATCTAACTGAGTGAGAGGTCAAGTTCTGGTGTGAAGATGATTGGTG  
 T L D S N P A T F P L N A T L Y A D F S  
 360

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1021 ACTTTGGACTCTAACCCAGCTACTTTCCCATTTGAACGCTACTTTGTACGCTGACTTCTCT
-----+-----+-----+-----+-----+-----+-----+-----+-----+
1080 TGAAACCTGAGATTGGGTCGATGAAAGGGTAACCTGCGATGAAACATGCGACTGAAGAGA

380 H D N T M I S I F F A L G L Y N G T K P
CACGACAACACTATGATATCTATTTTCTTCGCTTTGGGTTTGTACAACGGTACCAAGCCA
1081 -----+-----+-----+-----+-----+-----+-----+-----+-----+
1140 GTGCTGTTGTGATACTATAGATAAAAGAAGCGAAACCCAAACATGTTGCCATGGTTCGGT

400 L S T T S V E S I E E T D G Y S A S W T
TTGTCTACTACTTCTGTTGAATCTATTGAAGAACTGACGGTTACTCTGCTTCTTGGACT
1141 -----+-----+-----+-----+-----+-----+-----+-----+-----+
1200 AACAGATGATGAAGACAACCTTAGATAACTTCTTTGACTGCCAATGAGACGAAGAACCTGA

420 V P F A A R A Y V E M M Q C Q A E K E P
GTTCCATTGCTGCTAGAGCTTACGTTGAAATGATGCAATGTCAAGCTGAAAAGGAACCA
1201 -----+-----+-----+-----+-----+-----+-----+-----+-----+
1260 CAAGGTAAGCGACGATCTCGAATGCAACTTTACTACGTTACAGTTCGACTTTTCCTTGGT

440 L V R V L V N D R V V P L H G C A V D K
TTGGTTAGAGTTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTGCTGTTGACAAG
1261 -----+-----+-----+-----+-----+-----+-----+-----+-----+
1320 AACCAATCTCAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACACGACAACCTGTTT

460 L G R C K R D D F V E G L S F A R S G G
TTGGGTAGATGTAAGAGAGACGACTTCGTTGAAGGTTTGTCTTTTCGCTAGATCTGGTGGT
1321 -----+-----+-----+-----+-----+-----+-----+-----+-----+
1380 AACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGATCTAGACCACCA

N W A E C F A * 467
AACTGGGCTGAATGTTTCGCTTAA
1381 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1410
TTGACCCGACTTACAAAGCGAATT

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Figure 20

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      M G V F V V L L S I A T L F G S T S G T
20   ATGGGCGTGTTCGTGCTACTGTCCATTGCCACCTTGTTCCGGTCCACATCCGGTACC
      1 -----+-----+-----+-----+-----+
60   TACCCGCACAAGCAGCACGATGACAGGTAACGGTGGAACAAGCCAAGGTGTAGGCCATGG
      A L G P R G N S H S C D T V D G G Y Q C
40   GCCTTGGGTCCTCGTGGTAACTCTCACTCTTGTGACACTGTTGACGGTGGTTACCAATGT
      61 -----+-----+-----+-----+-----+
120  CGGAACCCAGGAGCACCATTGAGAGTGAGAACACTGTGACAACGCCACCAATGGTTACA
      A F P E I S H L W G T Y S P F F S L A D E
60   TTTCCAGAAATTTCTCACTTGTGGGGTACATACTCTCCATTCTTCTCTTTGGCTGACGAA
      121 -----+-----+-----+-----+-----+
180  AAGGGTCTTTAAAGAGTGAACACCCCATGTATGAGAGGTAAGAAGAGAAACCGACTGCTT
      S A I S P D V P K G C R V T F V Q V L S
80   TCTGCTATTTCTCCAGACGTTCCAAAGGGTTGTAGAGTTACTTTCGTTCAAGTTTGTCT
      181 -----+-----+-----+-----+-----+
240  AGACGATAAAGAGGTCTGCAAGGTTTCCCAACATCTCAATGAAAGCAAGTTCAAAACAGA
      R H G A R Y P T S S A S K A Y S A L I E
100  AGACACGGTGCTAGATACCCAATTCTTCTGCGTCTAAGGCGTACTCTGCTTTGATTGAA
      241 -----+-----+-----+-----+-----+
300  TCTGTGCCACGATCTATGGGTTGAAGAAGACGCAGATTCCGCATGAGACGAAACTAACTT
      A I Q K N A T A F K G K Y A F L K T Y N
120  GCTATTCAAAGAAGCCTACTGCTTTCAAGGGTAAGTACGCTTTCTTGAAGACTTACAAC
      301 -----+-----+-----+-----+-----+
360  CGATAAGTTTCTTGCATGACGAAAGTTCCCATTCATGCGAAAGAACTTCTGAATGTTG
      A Y T L G A D D L T P F G E Q Q M V N S G
140  TACACTTTGGGTGCTGACGACTTGACTCCATTCCGGTGAACAACAAATGGTTAACTCTGGT
      361 -----+-----+-----+-----+-----+
420  ATGTGAAACCCACGACTGCTGAACTGAGGTAAGCCACTTGTTGTTTACCAATTGAGACCA
      I K F Y R R Y K A L A R K I V P F I R A
160  ATTAAGTTCTACAGAAGATACAAGGCTTTGGCTAGAAAGATTGTTCCATTCTTAGAGCT
      421 -----+-----+-----+-----+-----+
480  TAATTCAAGATGTCTTCTATGTTCCGAAACCGATCTTTCTAACAAGGTAAGTAATCTCGA
      S G S D R V I A S A E K F I E G F Q S A
180  TCTGGTCTGACAGAGTTATTGCTTCTGCTGAAAAGTTCATTGAAGGTTTCCAATCTGCT

```

481 -----+-----+-----+-----+-----+-----+-----+  
 540 AGACCAAGACTGTCTCAATAACGAAGACGACTTTTCAAGTAACTCCAAAGGTTAGACGA  
 K L A D P G A N P H Q A S P V I N V I I  
 200 AAGTTGGCTGACCCAGGTGCTAACCACACCAAGCTTCTCCAGTTATTAACGTTATTATT  
 541 -----+-----+-----+-----+-----+-----+-----+  
 600 TTCAACCGACTGGGTCCACGATTGGGTGTGGTTCGAAGAGGTCAATAATTGCAATAATAA  
 P E G A G Y N N T L D H G L C T A F E E  
 220 CCAGAAGGTGCTGGTTACAACAACACTTTGGACCACGGTTTGTGTACTGCTTTCGAAGAA  
 601 -----+-----+-----+-----+-----+-----+-----+  
 660 GGTCTTCCACGACCAATGTTGTTGTGAAACCTGGTGCCAAACACATGACGAAAGCTTCTT  
 S E L G D D V E A N F T A V F A P P I R  
 240 TCTGAATTGGGTGACGACGTTGAAGCTAACTTCACTGCTGTTTTCGCTCCACCAATTAGA  
 661 -----+-----+-----+-----+-----+-----+-----+  
 720 AGACTTAACCCACTGCTGCAACTTCGATTGAAGTGACGACAAAAGCGAGGTGGTTAATCT  
 A R L E A H L P G V N L T D E D V V N L  
 260 GCTAGATTGGAAGCTCACTTGCCAGGTGTTAACTTGACTGACGAAGACGTTGTTAACTTG  
 721 -----+-----+-----+-----+-----+-----+-----+  
 780 CGATCTAACCTTCGAGTGAACGGTCCACAATTGAACTGACTGCTTCTGCAACAATTGAAC  
 M D M C P F D T V A R T S D A T Q L S P  
 280 ATGGACATGTGTCCATTGCACTGTTGCTAGAACTTCTGACGCTACTCAATTGTCTCCA  
 781 -----+-----+-----+-----+-----+-----+-----+  
 840 TACCTGTACACAGGTAAGCTGTGACAACGATCTTGAAGACTGCGATGAGTTAACAGAGGT  
 F C D L F T H D E W I Q Y D Y L Q S L G  
 300 TTCTGTGACTTGTTCCTCAGCAGCAATGGATTCAATACGACTACTTGAATCTTTGGGT  
 841 -----+-----+-----+-----+-----+-----+-----+  
 900 AAGACACTGAACAAGTGAGTGCTGCTTACCTAAGTTATGCTGATGAACGTTAGAAACCCA  
 K Y Y G Y G A G N P L G P A Q G V G F V  
 320 AAGTACTACGGTTACGGTGCTGGTAACCCATTGGGTCCAGCTCAAGGTGTTGGTTTCGTT  
 901 -----+-----+-----+-----+-----+-----+-----+  
 960 TTCATGATGCCAATGCCACGACCATTGGGTAACCCAGGTTCGAGTTCCACAACCAAAGCAA  
 N E L I A R L T H S P V Q D H T S T N H  
 340 AACGAATTGATTGCTAGATTGACTCACTCTCCAGTTCAAGACCACACTTCTACTAACCAC  
 961 -----+-----+-----+-----+-----+-----+-----+  
 1020 TTGCTTAACTAACGATCTAACTGAGTGAGAGGTCAAGTTCTGGTGTGAAGATGATTGGTG  
 T L D S N P A T F P L N A T L Y A D F S  
 360

```

1021 ACTTTGGACTCTAACCCAGCTACTTTCCCATTTGAACGCTACTTTGTACGCTGACTTCTCT
-----+-----+-----+-----+-----+-----+
1080 TGAAACCTGAGATTGGGTCGATGAAAGGGTAACCTGCGATGAAACATGCGACTGAAGAGA
H D N T M V S I F F A L G L Y N G T K P
380 CACGACAACACTATGGTTTCTATTTTCTTCGCTTTGGGTTTGTACAACGGTACTAAGCCA
1081 -----+-----+-----+-----+-----+-----+
1140 GTGCTGTTGTGATACCAAAGATAAAAGAAGCGAAACCCAAACATGTTGCCATGATTCGGT
L S T T S V E S I E E T D G Y S A S W T
400 TTGTCTACTACTTCTGTTGAATCTATTGAAGAACTGACGGTTACTCTGCTTCTTGGACT
1141 -----+-----+-----+-----+-----+-----+
1200 AACAGATGATGAAGACAACTTAGATAACTTCTTTGACTGCCAATGAGACGAAGAACCTGA
V P F A A R A Y V E M M Q C E A E K E P
420 GTTCCATTGCTGCTAGAGCTTACGTTGAAATGATGCAATGTGAAGCTGAAAAGGAACCA
1201 -----+-----+-----+-----+-----+-----+
1260 CAAGGTAAGCGACGATCTCGAATGCAACTTTACTACGTTACACTTCGACTTTTCCTTGGT
L V R V L V N D R V V P L H G C G V D K
440 TTGGTTAGAGTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTGGTGTGACAAG
1261 -----+-----+-----+-----+-----+-----+
1320 AACCAATCTCAAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACACCACAACCTGTTC
L G R C K R D D F V E G L S F A R S G G
460 TTGGGTAGATGTAAGAGAGACGACTTCGTTGAAGGTTTGTCTTTCGCTAGATCTGGTGGT
1321 -----+-----+-----+-----+-----+-----+
1380 AACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGATCTAGACCACCA
N W E E C F A * 467
AACTGGGAAGAATGTTTCGCTTAA
1381 -----+-----+-----+-----+-----+ 1404
TTGACCCTTCTTACAAAGCGAATT

```



Figure 21

[illegible]

541 GCGAAGCTGGCTGATCCTGGCGCGACGAACCGCGCCGCTCCGGCGATTAGTGTGATTATT  
 600 CGCTTCGACCGACTAGGACCGCGCTGCTTGGCGCGGCGAGGCCGCTAATCACACTAATAA  
 P E S E T F N N T L D H G V C T K F E A  
 220 CCGGAGAGCGAGACGTTCAACAATACGCTGGACCACGGTGTGTGCACGAAGTTTGAGGCG  
 601 GGCCTCTCGCTCTGCAAGTTGTTATGCGACCTGGTGCCACACACGTGCTTCAAACCTCCGC  
 660 S Q L G D E V A A N F T A L F A P D I R  
 240 AGTCAGCTGGGAGATGAGGTTGCGGCCAATTTCACTGCGCTCTTTGCACCCGACATCCGA  
 661 TCAGTCGACCCCTCTACTCCAACGCCGGTTAAAGTGACGCGAGAAACGTGGGCTGTAGGCT  
 720 A R L E K H L P G V T L T D E D V V S L  
 260 GCTCGCctCGAGAAGCATCTTCTGGCGTGACGCTGACAGACGAGGACGTTGTGCTAGTCTA  
 721 CGAGCGgaGCTCTTCGTAGAAGGACCGCACTGCGACTGTCTGCTCTGCAACAGTCAGAT  
 780 M D M C P F D T V A R T S D A S Q L S P  
 280 ATGGACATGTGTcCGTTTGATACGGTAGCGCGCACCAGCGACGCAAGTCAGCTGTACCCG  
 781 TACCTGTACACagGCAAACCTATGCCATCGCGCGTGGTCGCTGCGTTTCAGTCGACAGTGGC  
 840 F C Q L F T H N E W K K Y D Y L Q S L G  
 300 TTCTGTCAACTCTTCACTCACAATGAGTGAAGAAGTACgACTACCTTCAGTCCTTGGGC  
 841 AAGACAGTTGAGAAGTGAGTGTACTCACCTTCTTCATGcTGATGGAAGTCAGGAACCCG  
 900 K Y Y G Y G A G N P L G P A Q G I G F T  
 320 AAGTACTACGGCTACGGCGCAGGCAACCCCTCTGGGACCGGCTCAGGGGATAGGGTTCACC  
 901 TTCATGATGCCGATGCCCGCTCCGTTGGGAGACCCTGGCCGAGTCCCTTATCCCAAGTGG  
 960 N E L I A R L T R S P V Q D H T S T N S  
 340 AACGAGCTGATTGCCCGGTTGACgCGTTGCCAGTGACGAGGACACACCAGCACTAACTCG  
 961 TTGCTCGACTAACGGGCCAACTGcGCAAGCGGTACGTCCTGGTGTGGTGGTGGTATTGAGC  
 1020 T L V S N P A T F P L N A T M Y V D F S  
 360 ACTCTAGTCTCCAACCCGGCCACCTTCCCCTTGAACGCTACCATGTACGCTGACTTTTCA  
 1021 TGAGATCAGAGGTTGGGCCGGTGAAGGGCAACTTGCGATGGTACATGCAGCTGAAAAGT  
 1080

```

      H D N S M V S I F F A L G L Y N G T E P
380  CACGACAACAGCATGGTTTCCATCTTCTTGCATTGGGCCTGTACAACGGCACTGAACCC
1081 -----+-----+-----+-----+-----+
1140  GTGCTGTTGTCTGTACCAAAGGTAGAAGAAACGTAACCCGGACATGTTGCCGTGACTTGGG
      L S R T S V E S A K E L D G Y S A S W V
400  TTGTCCCGGACCTCGGTGGAAGCGCCAAGGAATTGGATGGGTATTCTGCATCCTGGGTG
1141 -----+-----+-----+-----+-----+
1200  AACAGGGCCTGGAGCCACCTTTCGCGGTTTCCTTAACCTACCCATAAGACGTAGGACCCAC
      V P F G A R A Y F E T M Q C K S E K E P
420  GTGCCTTTCGGCGCGGAGCCTACTTCGAGACGATGCAATGCAAGTCGGAAGGAGCCT
1201 -----+-----+-----+-----+-----+
1260  CACGGAAAGCCGCGCGCTCGGATGAAGCTCTGCTACGTTACGTTACGCTTTTCCTCGGA
      L V R A L I N D R V V P L H G C D V D K
440  CTTGTTGCGGCTTTGATTAATGACCGGTTGTGCCACTGCATGGCTGCGATGTGGACAAG
1261 -----+-----+-----+-----+-----+
1320  GAACAAGCGCGAACTAATTACTGGCCCAACACGGTGACGTACCGACGCTACACCTGTTC
      L G R C K L N D F V K G L S W A R S G G
460  CTGGGGCGATGCAAGCTGAATGACTTTGTCAAGGGATTGAGTTGGGCCAGATCTGGGGGC
1321 -----+-----+-----+-----+-----+
1380  GACCCCGCTACGTTGACTTACTGAAACAGTTCCTAACTCAACCCGGTCTAGACCCCGG
      N W G E C F S * 467
      AACTGGGGAGAGTGCTTTAGTTGA
1381 -----+-----+-----+-----+-----+ 1404
      TTGACCCCTCTCACGAAATCAACT

```

Figure 22

CP-1  
 Eco RI M G V F V V L L S I A T L F G S T  
 TATATGAATTCATGGCGTGTTCGTCGTGCTACTGTCCATTGCCACCTTGTTCGGTTCCA  
 1 -----+-----+-----+-----+-----+ 60  
 ATATACTTAAGTACCCGCACAAGCAGCAGCATGACAGGTAACGGTGAACAAGCCAAGGT  
  
 S G T A L G P R G N S H S C D T V D G G  
 CATCCGGTACCGCCTTGGGTCTCGTGGTAATTCTCACTCTTGTGACACTGTTGACGGTG  
 61 -----+-----+-----+-----+-----+  
 120 GTAGGCCATGGCGGAACCCAGGAGCACCATTAAAGAGTGAGAACAACCTGTGACAACCTGCCAC  
 CP-2  
 CP-3  
 Y Q C F P E I S H L W G Q Y S P Y F S L  
 GTTACCAATGTTTCCAGAAATTTCTCACTTGTGGGGTCAATACTCTCCATACTTCTCTT  
 121 -----+-----+-----+-----+-----+  
 180 CAATCGTTACAAAGGGTCTTTAAAGAGTGAACACCCCAGTTATGAGAGGTATGAAGAGAA  
  
 E D E S A I S P D V P D D C R V T F V Q  
 TGGAAGACGAATCTGCTATTTCTCCAGACGTTCCAGACGACTGTAGAGTTACTTTCGTTT  
 181 -----+-----+-----+-----+-----+  
 240 ACCTTCTGCTTAGACGATAAAGAGGTCTGCAAGGTCTGCTGACATCTCAATGAAAGCAAG  
 CP-4.7  
 CP-5.7  
 V L S R H G A R Y P T D S K G K K Y S A  
 AAGTTTGTCTAGACACGGTGTAGATACCCAACtGacTCTAAGggtAAGaagTACTCTG  
 241 -----+-----+-----+-----+-----+  
 300 TTCAAACAGATCTGTGCCACGATCTATGGGTGActgAGATTCCcattCttcATGAGAC  
  
 L I E A I Q K N A T A F K G K Y A F L K  
 CTTTGATTGAAGCTATTCAAAGAAGCGTACTGCTTTCAAGGGTAAGTACGCTTTCTTGA  
 301 -----+-----+-----+-----+-----+  
 360 GAAACTAACTTCGATAAGTTTTCTTTCGATGACGAAAGTTCCCATTCATGCGAAAGAAGT  
 CP-6  
 CP-7  
 T Y N Y T L G A D D L T P F G E N Q M V  
 AGACTTACAACTACACTTTGGGTGCTGACGACTTGACTCCATTCCGGTGAAAACCAAATGG  
 361 -----+-----+-----+-----+-----+  
 420 TCTGAATGTTGATGTGAAACCCACGACTGCTGAACTGAGGTAAGCCACTTTTGGTTTACC  
  
 N S G I K F Y R R Y K A L A R K I V P F  
 TTAACCTCTGCTATTAAAGTTCTACAGAAGATAACAAGCCTTTGGCTAGAAAGATTGTTCCAT  
 421 -----+-----+-----+-----+-----+  
 480 AATTGAGACCATAATTCAAGATGTCTTCTATGTTCCGAAACCGATCTTTCTAACAAGGTA  
 CP-8.7  
 CP-9  
 I R A S G S S R V I A S A E K F I E G F  
 TCATTAGAGCTTCTGGTCTTctAGAGTTATTGCTTCTGCTGAAAAGTTTATTGAAGGTT  
 481 -----+-----+-----+-----+-----+  
 540 AGTAATCTCGAAGACCAAGAagaTCTCAATAACGAAGACGACTTTTCAAGTAACCTCCAA  
  
 Q S A K L A D P G S Q P H Q A S P V I D  
 TCCAATCTGCTAAGTTGGCTGACCCAGGTTCTCAACCACACCAAGCTTCTCCAGTTATTG

541 -----+-----+-----+-----+-----+-----+-----+  
600 AGGTTAGACGATTCAACCGACTGGGTCCAAGAGTTGGTGTGGTTCTGAAGAGGTCAATAAC  
CP-10.7  
V I I S E A S S Y N N T L D P G T C T A  
CP-11.7  
ACGTTATTATTtctGAcgctTCTtctTACAACAACACTTTGGACccaGGTACTTGTACTG  
601 -----+-----+-----+-----+-----+-----+-----+  
660 TGCAATAATAAagaCTgcgaAGGagaATGTTGTTGTGAAACCTGggtCCATGAACATGAC

F E D S E L A D T V E A N F T A L F A P  
 CTTTCGAAGACTCTGAATTGgctGACactGTTGAAGCTAACTTCACTGCTTTGTTGCTC  
 661 -----+-----+-----+-----+-----+-----+-----+-----+-----+  
 720  
 GAAAGCTTCTGAGACTTAACcgaCTGtgacAACTTCGATTGAAGTGACGAAACAAGCGAG  
 CP-12.7  
 A I R A R L E A D L P G V T L T D T E V  
 CAGCTATTAGAGCTAGATTGGAAGCTGACTTGCCAGGTGTTACTTTGACTGACactgaaG  
 721 -----+-----+-----+-----+-----+-----+-----+-----+-----+  
 780  
 GTCGATAATCTCGATCTAACCTTCGACTGAACGGTCCACAATGAACTGACTGtgacttc  
 CP-13.7  
 T Y L M D M C S F E T V A R T S D A T E  
 TTactTACTTGATCGACATGTGTtctTTCGAACTGTTGCTAGAACTTCTGACGCTACTG  
 781 -----+-----+-----+-----+-----+-----+-----+-----+-----+  
 840  
 AatgaATGAACTACCTGTACACAagaAAGCTTTGACAACGATCTTGAAGACTGCGATGAC  
 L S P F C A L F T H D E W R H Y D Y L Q  
 AATGTCTCCATTCTGTGCTTTGTTCACTCACGACGAATGGAGAcacTACGACTACTTGC  
 841 -----+-----+-----+-----+-----+-----+-----+-----+-----+  
 900  
 TTAACAGAGGTAAGACACGAAACAAGTGAGTGCTGCTTACCTCTgtgATGCTGATGAACG  
 CP-14.7  
 CP-15.7  
 S L K K Y Y G H G A G N P L G P T Q G V  
 AATCTTTGaagAAGTACTACGGTcacGGTGCTGGTAACCCATTGGGTCCAactCAAGGTG  
 901 -----+-----+-----+-----+-----+-----+-----+-----+-----+  
 960  
 TTAGAAActtcTTCATGATGCCAgtgCCACGACCATTGGGTAACCCAGGTTgaGTTCCAC  
 G F A N E L I A R L T R S P V Q D H T S  
 TTGGTTTCGCTAACGAATTGATTGCTAGATTGACTAGATCTCCAGTTCAAGACCACACTT  
 961 -----+-----+-----+-----+-----+-----+-----+-----+-----+  
 1020  
 AACCAAAGCGATTGCTTAACTAACGATCTAACTGATCTAGAGGTCAAGTTCTGGTGTGAA  
 CP-16  
 CP-17.7  
 T N H T L D S N P A T F P L N A T L Y A  
 CTACTAACCACTTTGGACTCTAACCCAGCTACTTTCCCATTTGAACGCTACTTTGTACG  
 1021 -----+-----+-----+-----+-----+-----+-----+-----+-----+  
 1080  
 GATGATTGGTGTGAAACCTGAGATTGGGTGCGATGAAAGGGTAACTTGCGATGAAACATGC  
 D F S H D N G I I S I F F A L G L Y N G  
 CTGACTTCTCTCACGACAACggtattATTCTATTTCTTCGCTTTGGGTTGTACAACG  
 1081 -----+-----+-----+-----+-----+-----+-----+-----+-----+  
 1140  
 GACTGAAGAGAGTGCTGTTGccataaTAAAGATAAAAGAAGCGAAACCCAAACATGTTGC  
 CP-18.7  
 CP-19.7  
 T A P L S T T S V E S I E E T D G Y S S  
 GTACTGCTCCATTGTCTACTACTTCTGTTGAATCTATTGAAGAACTGACGGTTACTCTt  
 1141 -----+-----+-----+-----+-----+-----+-----+-----+-----+  
 1200  
 CATGACGAGGTAACAGATGATGAAGACAACCTTAGATAACTTCTTTGACTGCCAATGAGaa  
 A W T V P F A S R A Y V E M M Q C Q A E

```

ctgctTGGACTGTTCCATTGctttctAGAGCTTACGTTGAAATGATGCAATGTCAAGCTG
1201 -----+-----+-----+-----+-----+-----+
1260 gacgaACCTGACAAGGTAAGcgaagaTCTCGAATGCAACTTTACTACGTTACAGTTTCGAC
                                     CP-20
                                     CP-21
      K E P L V R V L V N D R V V P L H G C A
1261 AAAAGGAACCATTGGTTAGAGTTTGGTTAACGACAGAGTTGTTCCATTGCACGGTTGTG
-----+-----+-----+-----+-----+-----+
1320 TTTTCCTTGGTAACCAATCTCAAAACCAATTGCTGTCTCAACAAGGTAACGTGCCAACAC

```

V D K L G R C K R D D F V E G L S F A R  
 CTGTTGACAAGTTGGGTAGATGTAAGAGAGACGACTTCGTTGAAGGTTTGTCTTTCGCTA  
 1321 -----+-----+-----+-----+-----+-----+-----+  
 1380 GACAACTGTTCAACCCATCTACATTCTCTCTGCTGAAGCAACTTCCAAACAGAAAGCGAT  
 CP-22  
 S G G N W A E C F A \* Eco RI  
 GATCTGGTGGTAACTGGGCTGAATGTTTCGCTTAAGAATTCATATA  
 1381 -----+-----+-----+-----+-----+-----+-----+ 1426  
 CTAGACCACCATTGACCCGACTTACAAAGCGAATTCTTAAGTATAT



Figure 23

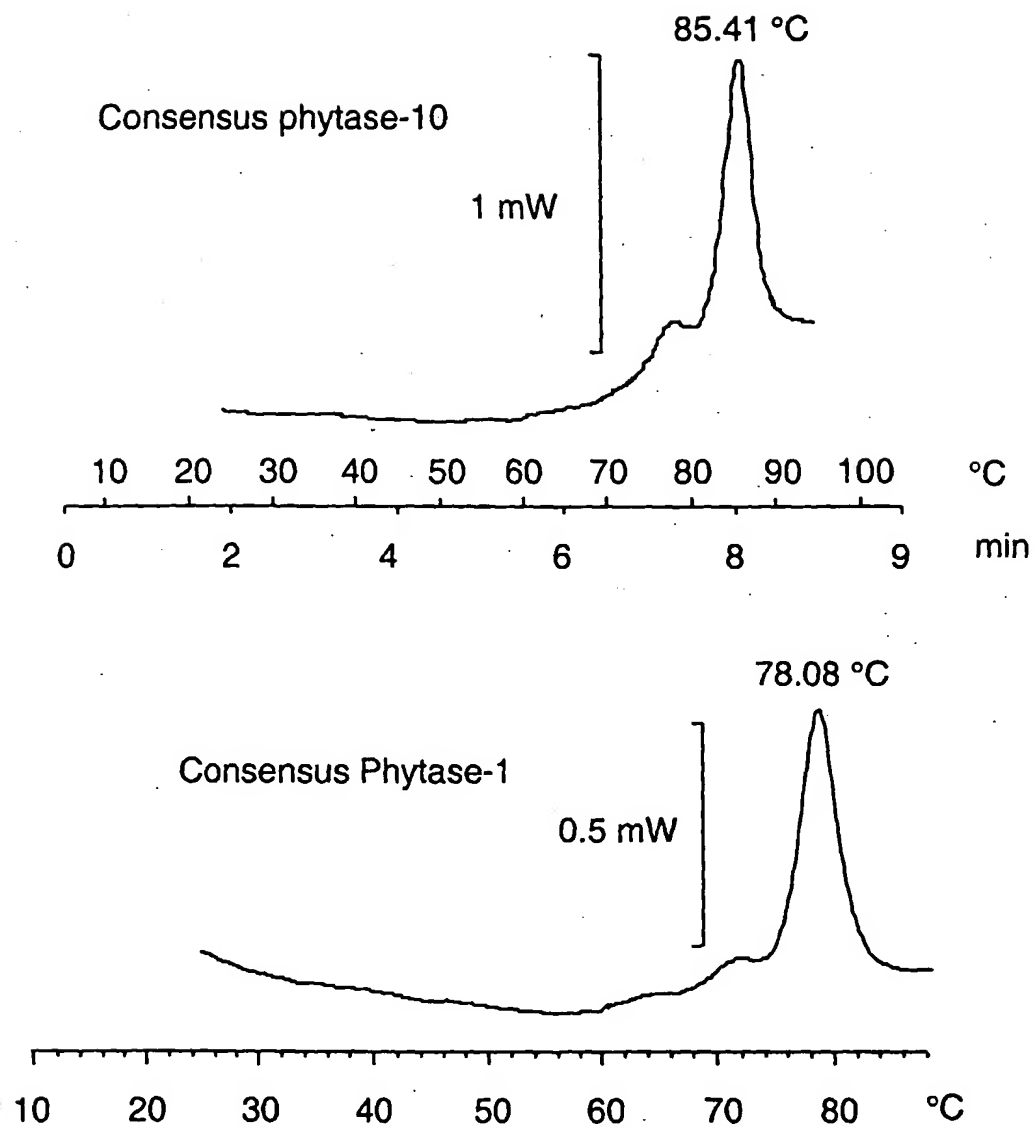


Figure 24

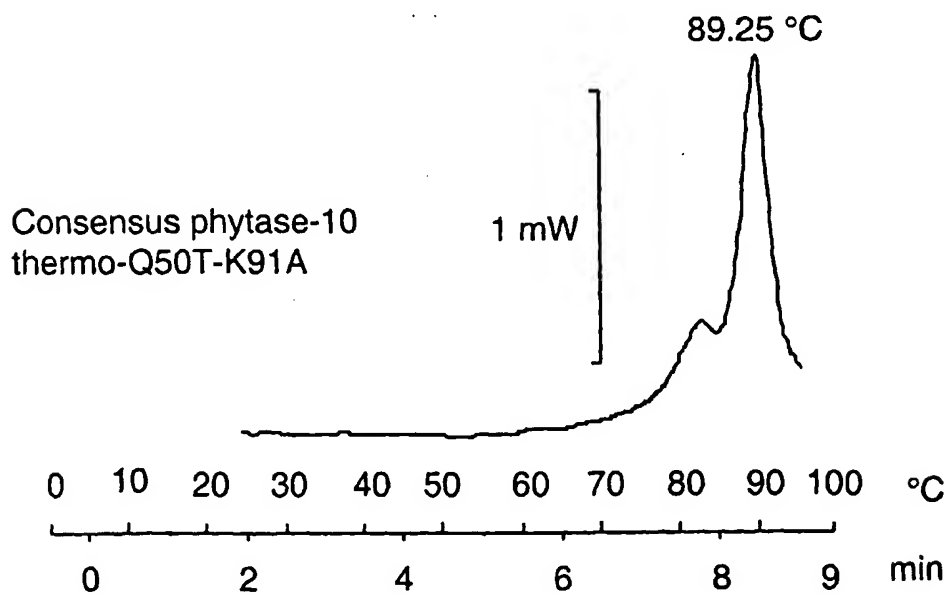
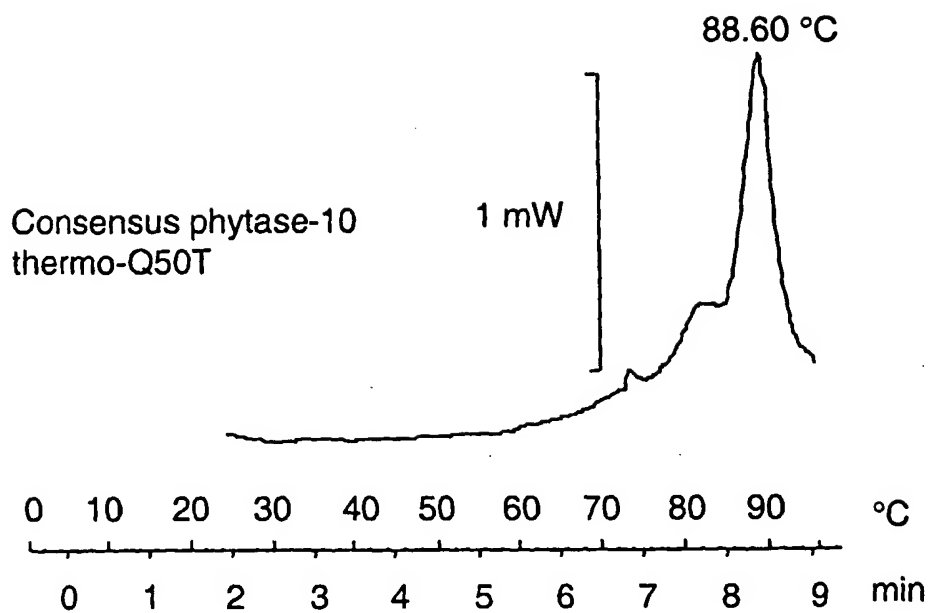


Figure 25

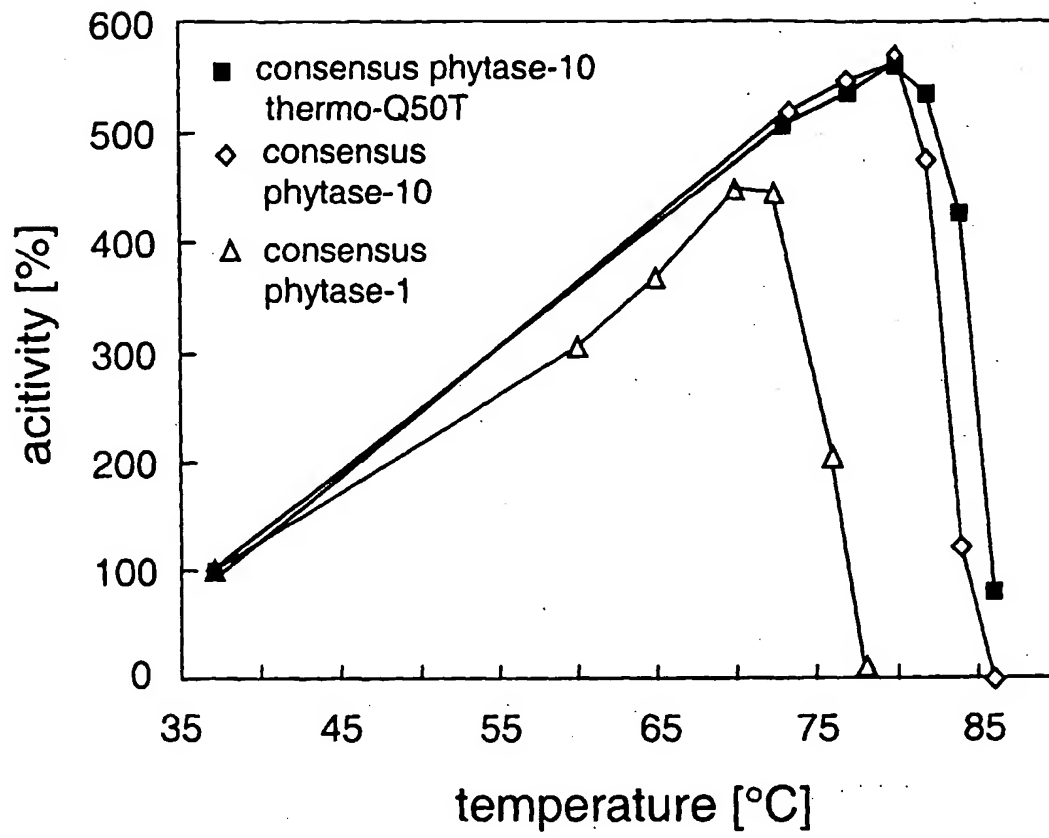


Figure 26

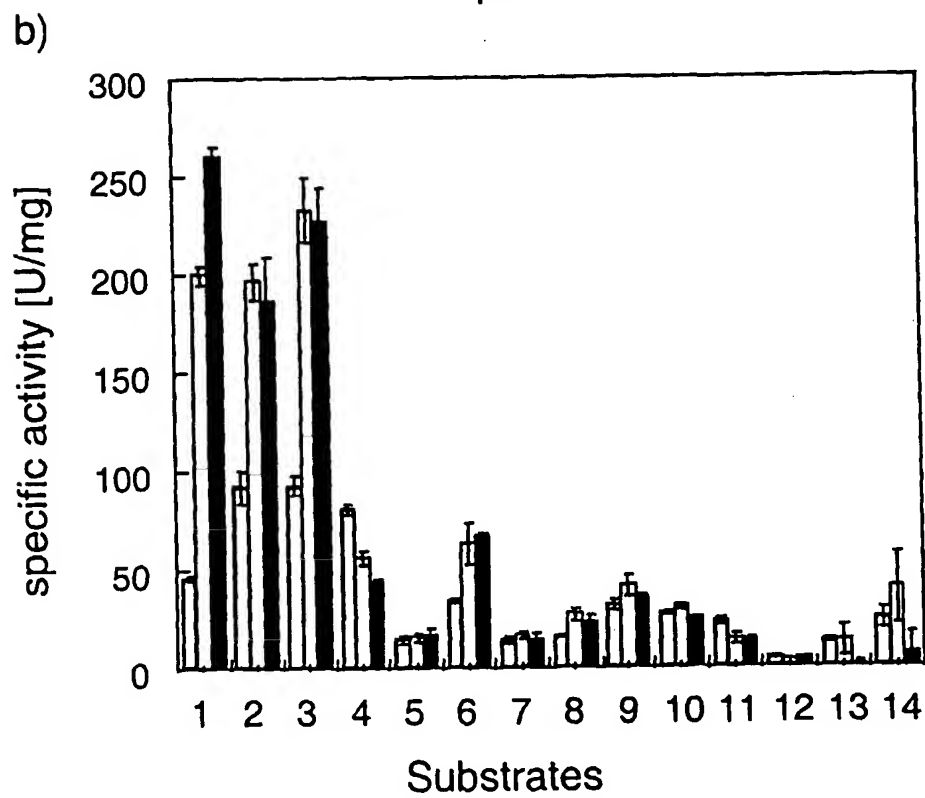
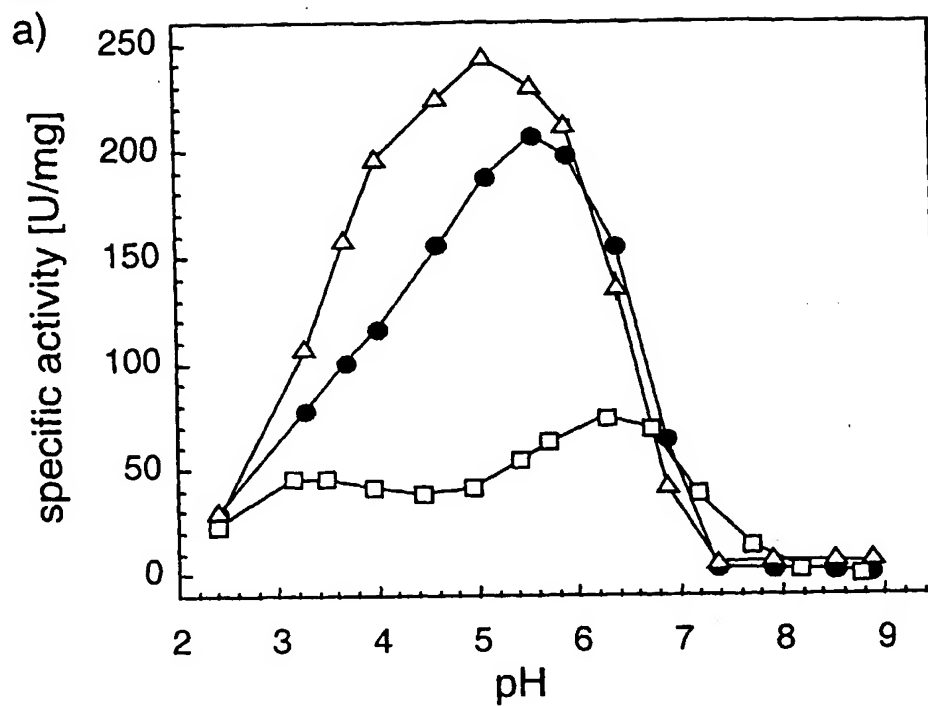


Figure 27

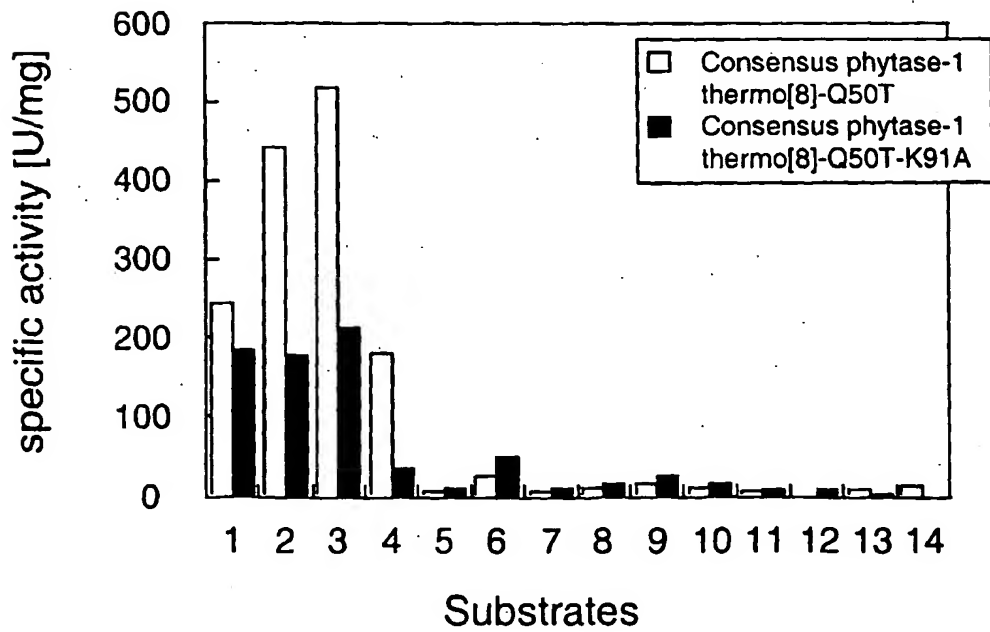
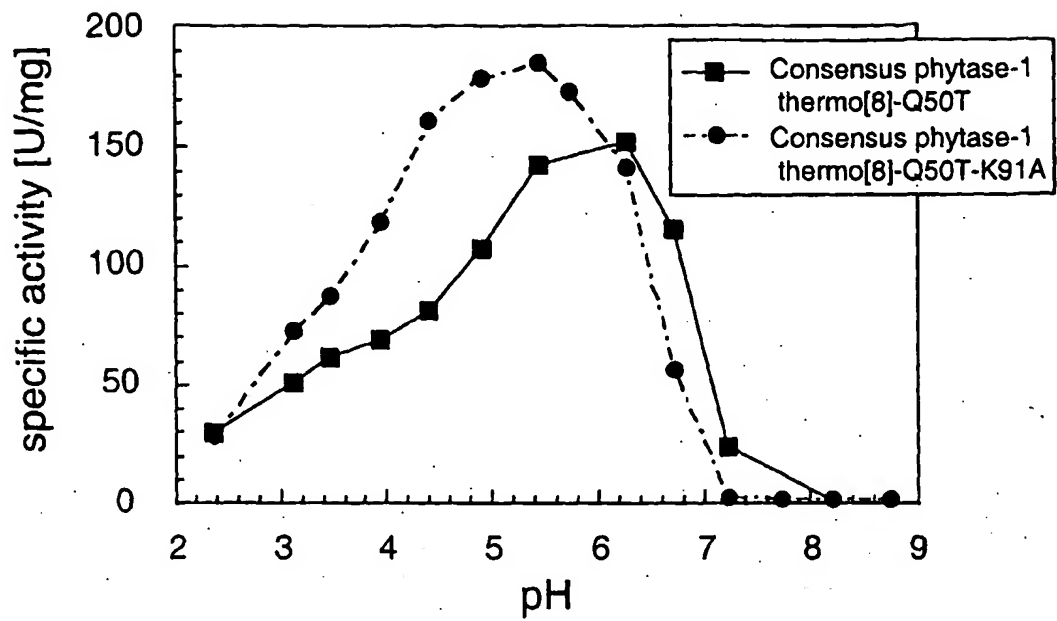


Figure 28

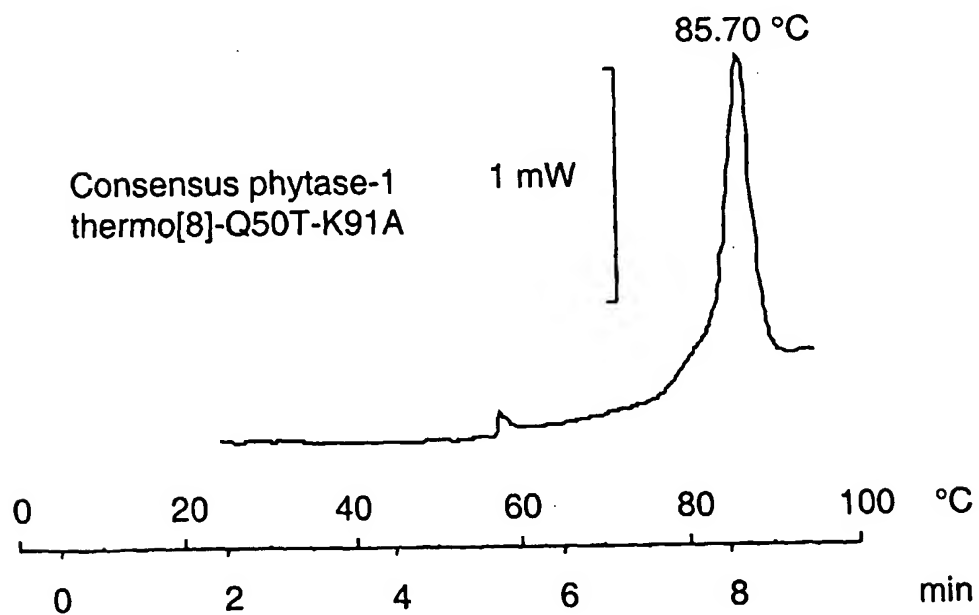
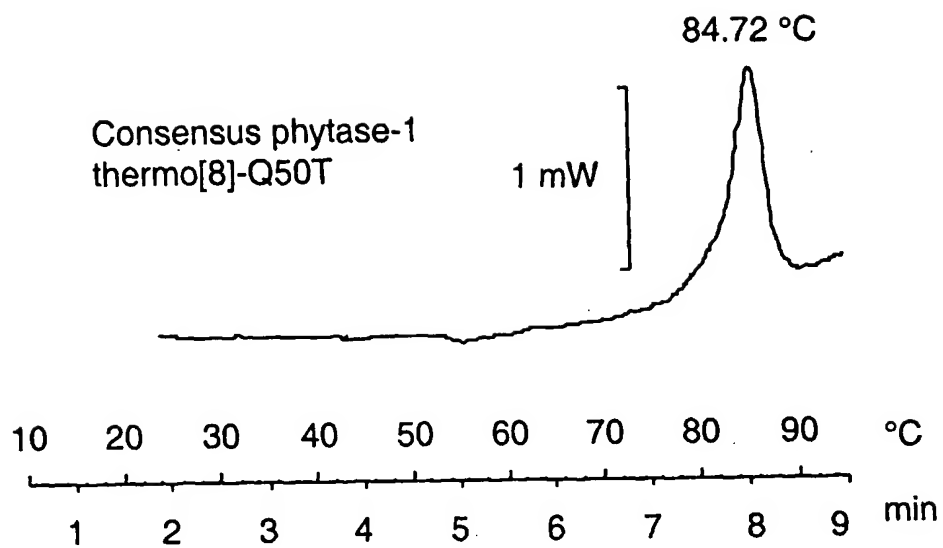


Figure 29

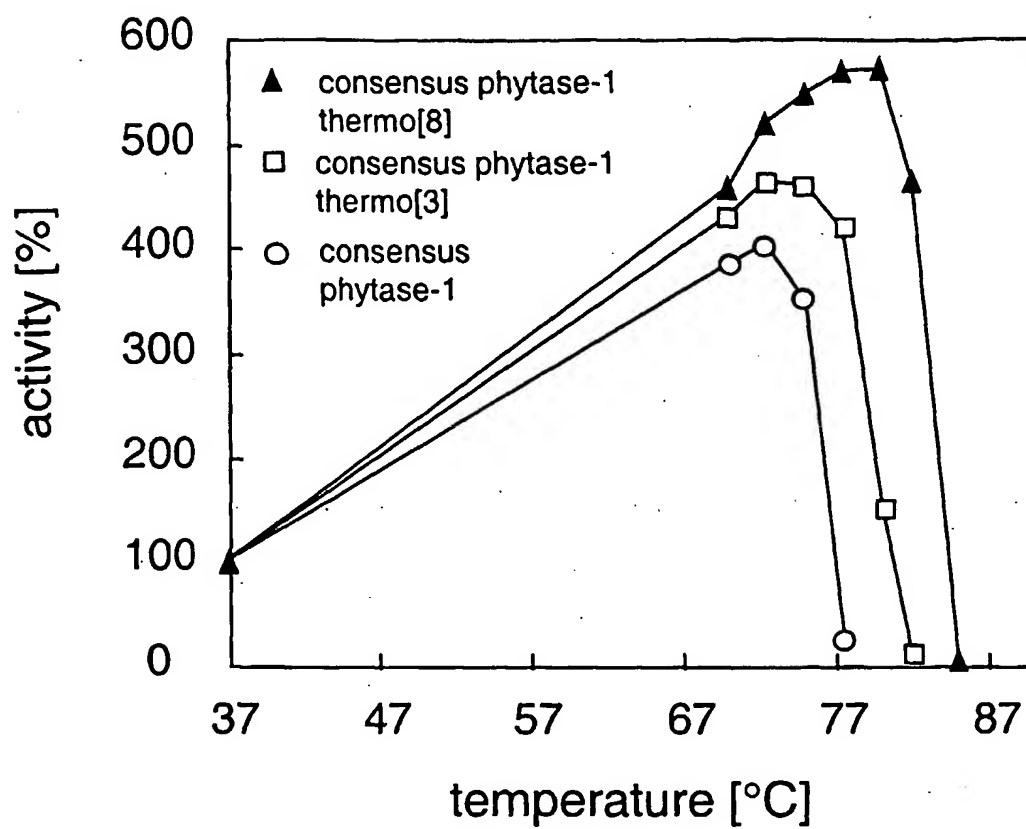


Figure 30

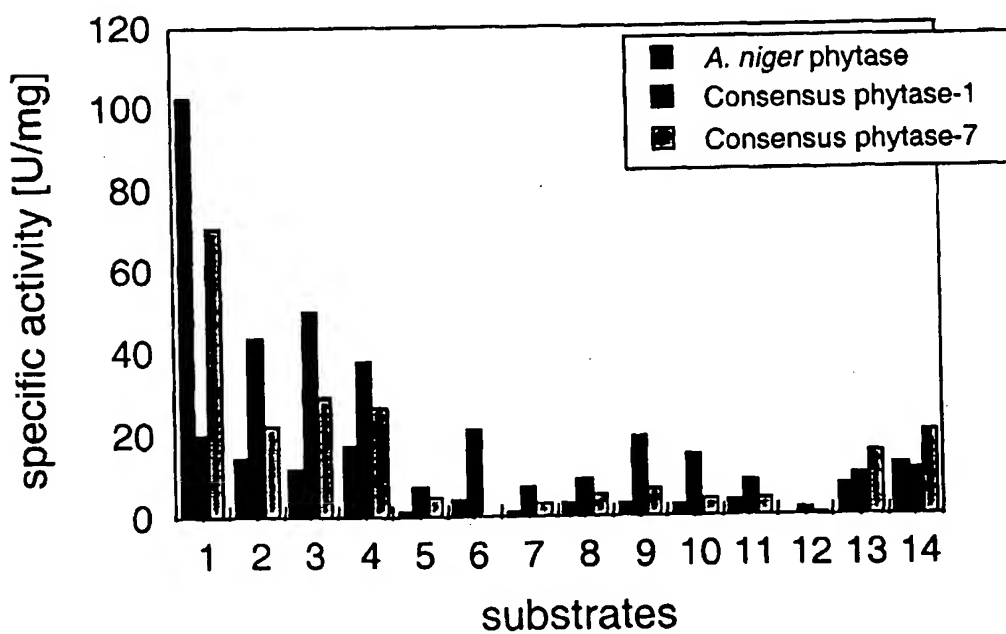
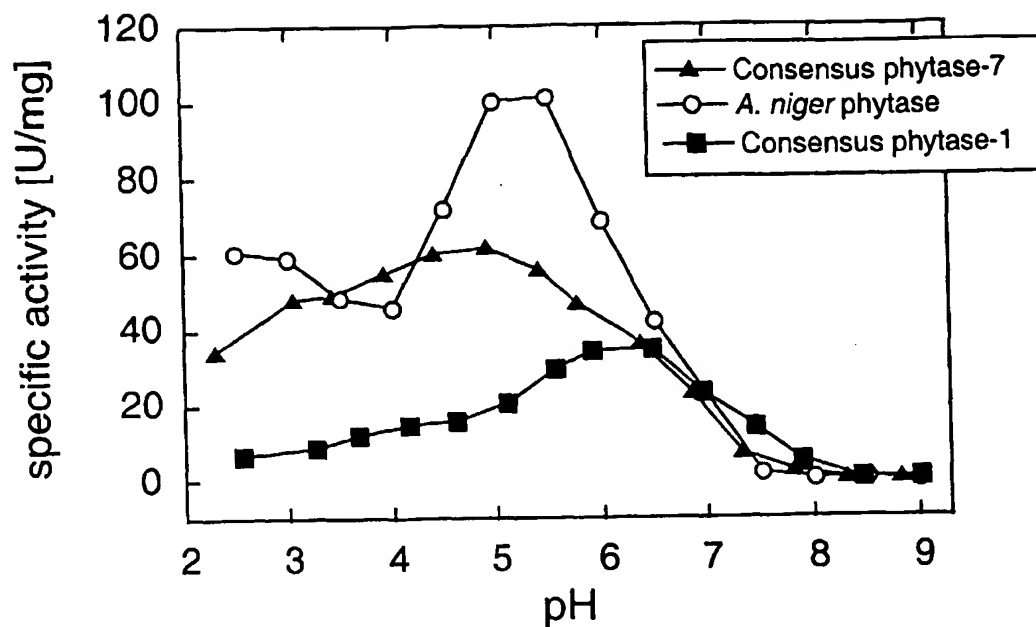




Figure 31

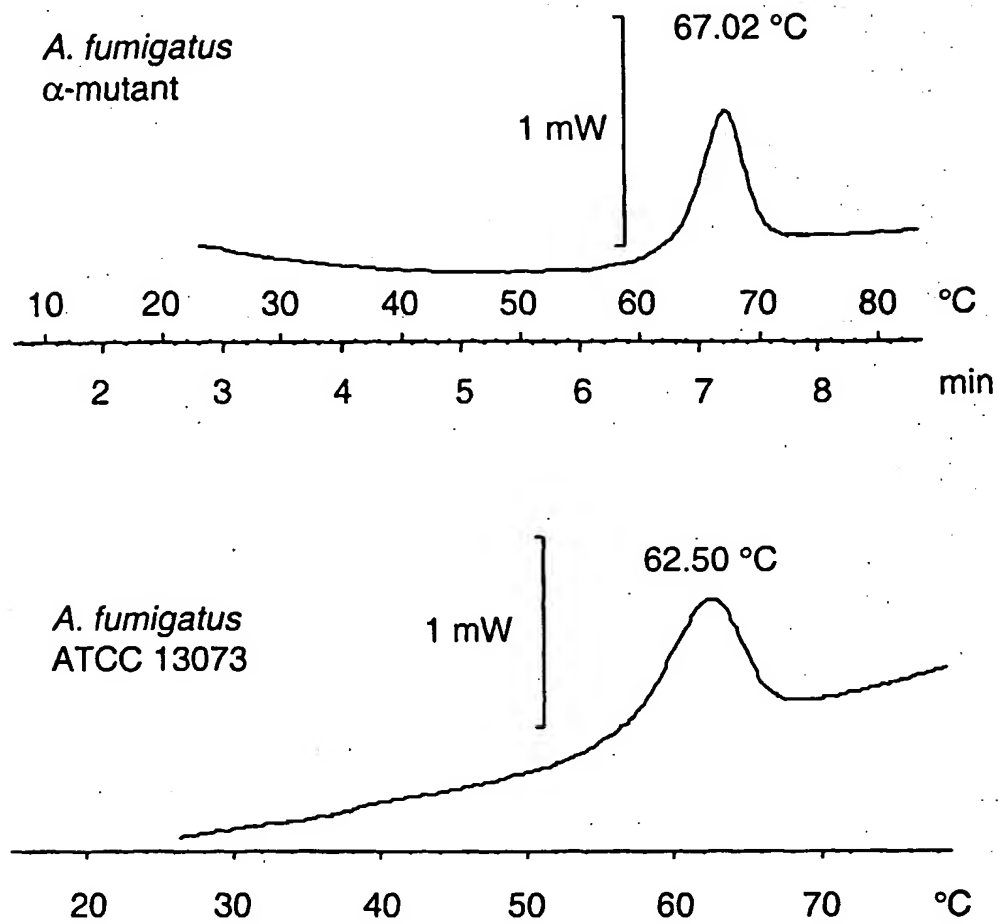


Figure 32

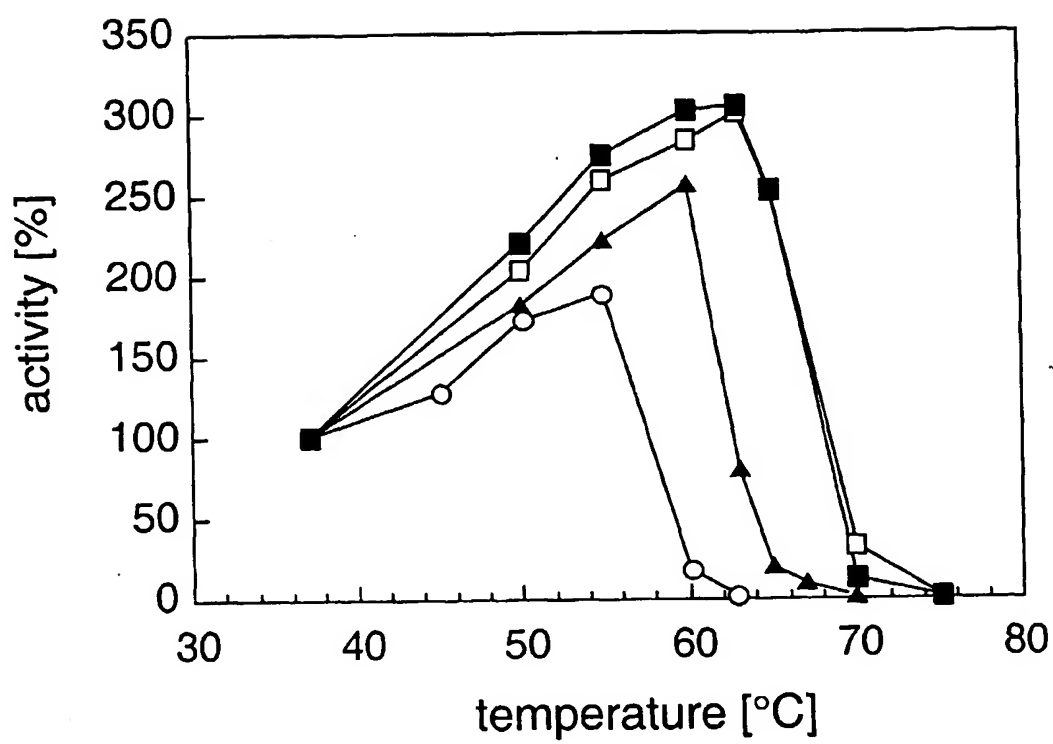


Figure 33

1 MGVFVLLSI ATLFGSTSGT ALGPRGNSHS CDTVGGYQC FPEISSNWSP  
 51 YSPYFSLADE SAISPDVPKG CRVTFVQVLQ RHGARFPTSG AATRISALIE  
 101 AIQKNATAFK GKYAFLKTYN YTLGADDLVP FGANQSSQAG IKFYRRYKAL  
 151 ARKIVPFIRA SGSDRVIDSA TNWIEGFQSA KLADPGANPH QASPVINVII  
 201 PEGAGYNNTL DHGLCTAFEE SELGDDVEAN FTAVFAPPIR ARLEAHLPGV  
 251 NLTDDEVVNL MDMCPFDIVA RTSDATELSP FCDLFTHDEW IQYDYLGDLD  
 301 KYYGIGAGNP LGPAQGVGVFV NELIARLTHS PVQDHTSTNH TLDSNPATFP  
 351 LNATLYADFS HDNTMVAIFF ALGLYNGTKP LSTTSVESIE ETDGYSASWL  
 401 VPFSARMYVE MMQCEAEKEP LVRVLVNDRV VPLHGCGVDK LGRCKRDDFV  
 451 EGLSFARSGG NWEECFA



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number  
EP 99 11 1949

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
P, X	WO 98 54980 A (HARZ HANS PETER ; BARENDSE RUDOLF CAROLUS MARIA (NL); GIST BROCADES) 10 December 1998 (1998-12-10) see page 12, lines 14-21; page 13, last paragraph; page 15, last paragraph; ---	1, 4, 15, 19, 20, 22	C12N9/96 C12N9/16 A23K1/165
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